

Sustainable Development and Biodiversity 1

Dinesh K. Maheshwari *Editor*

Bacterial Diversity in Sustainable Agriculture



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Editor

Bacterial Diversity in Sustainable Agriculture



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Preface

Agricultural sustainability requires a major slave from ecosystem management which is better paid by microbial diversity in general and bacterial diversity in particular. Diversity of bacteria influences crop productivity providing nutrient convenience from soil instead altering per se community and diversity in the rhizosphere where they may influence mechanistic component with deleterious microflora.

Bacteria are minute, pervasive in nature and alleged as disease host instead of being tiny organisms and are recognized as employee of agro-biological ecosystem indulge in agricultural development. The diverse nature proved them as potential contributors in world of ecological and economical wealth creation. This step pertinently may help to launch the scientific motivation required for support to refrain of microbial diversity and conservation.

The present book has 13 chapters that cover various facets of current scientific scenario on bacterial diversity that are associated with agro-ecosystem, so as to benefit plants for sustainable agriculture. The agro-ecosystem enriched with rhizosphere implicit abundant and species-rich component of beneficial diversity. A due account is provided with respect to global biological significance of diversity of actinobacteria, rhizobia and bacteria from vermicompost, which have an ecological significance. Besides natural microorganisms, role of cold tolerant bacteria from Himalayan region and osmotolerant from coastal region have suitably described. Transgenic Bt cotton on the soil microbial diversity and other related functions has also been included. Microbial population in agro-ecosystem is essential and is subjective to their maintenance for use around the globe. It is therefore, important to explore secrets of culture independent diversified microbial communities for improvement in agricultural system with economically sound production of human food and animal feed.

This book will be useful for students, teachers and researchers but also to those interested to strengthen subjects of microbiology, ecology, phytopathology, physiology, environmental biology and NGO's working for the protection of species, loss of genetic material and consequently overall agricultural sustainability.

I would like to express my sincere thanks to all the subject experts for their much needed co-operation authoritative and up to date information organized in a befitting manner. I acknowledge with thanks the assistance rendered by my Ph.D.

students Shrivardhan, Mohit, Chitra and Garima. I am also thankful to University Grant Commission, New Delhi and UCOST, Dehradun for their support in execution of my research projects on microbial diversity as a prelude to lay foundation for compilation of the volume like this. I extend thanks to Dr. Valeria Rinaudo from the publisher Springer for her valuable support. Last but not the least, I owe thanks to my wife Dr. Sadhana and my children Dr. Charul and Ashish for taking care of me during this project.

‘Basant Panchmi’
Haridwar, India

Dinesh K. Maheshwari

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Chapter 1

Trends and Prospects of Microbial Diversity in Rhizosphere

Dinesh Kumar Maheshwari, Mohit Agarwal and Shrivardhan Dheeman

Abstract Biodiversity is an appealing aspect including microorganisms encompasses diverse assemblage of organisms exhibiting with morphological, physiological, biochemical, metabolic and ecological diversity. At the level of taxonomic diversity microorganism signify phenotypic and genotypic diversity. Whereas, phenotypic diversity physiologically diversify the population of bacteria on the other hand, genotypic diversity explore the genetic divergence within the bacterial population. Functional diversity in the rhizosphere in particular is also considered as functional behavior of microbial community in their ecological niche or ecosystem. Functional diversity is the measures of distribution and functional niche of organisms where they remain functional in communities and multi-dimensional cloud of species trait and each trait representing an individual or a species. Most often define biodiversity as the “totality of genes, species, and ecosystems of a region studied under term species diversity, genetic diversity and ecological diversity.” Microbial diversity in rhizosphere bears several interactions and controls the functional roles to permit parse and plant survival. Measurement of biodiversity on species richness and evenness/dominance indices, species abundance models is recent tools to assess microbial diversity. Rhizosphere implicit microbial population aids benefits for plants in term of growth promotion and proved itself as important and putative communities for increased crop productivity.

1.1 Introduction

The term biodiversity was used first by Wilson (1988). Since then the concept of biodiversity continuous spread to arose as developing and emerging field. Biodiversity is defined as the variety of living organisms at species, inter-species and intra-species level in ecology which is much considered in terms of plants and animals; yet, the assortment of microbial life forms a huge and largely uncharted account. Microorganisms are quite beneficial and exploited in gain of quite lot revenue for

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humankind and earth's ecology. Similarly, as the plants and animals are conserved to counter the alarm of extinction of their species, the great reservoir of rhizospheric microbial diversity, employed for ecological and humankind services should be conserved in the means of culture collection and genomic library construction and utilization in agriculture. Such appealing organisms having distributional merit in all three dimensions of nature and ecology viz., soil, water and air, where microbial diversity signify at distinct ecological levels. The soil is a reservoir and prime nutritional habitat of severe community of phenotypic and genotypic diverse microorganisms. It is daunting task to grasp considerate of microbial diversity so, under this quest, it is necessary to group such organisms together so as to organize them in non-overlapping hierarchy. On account of classification system using polyphasic taxonomy i.e., phenotypic, genotypic and phylogenetic classification results diversity at hierarchical level. Microbial diversity also saturate on other three levels of biodiversity which are species diversity, genetic diversity and ecological diversity. Measurement of diversity looks on their entire structure and defined on virtue of proximity and remoteness in number of trait, of individuals, or species in its ecology by employing at all three levels. Under this review article, emphasis will be drawn on (i) understanding of microbial diversity of rhizospheric ecology and its measurement (ii) application of versatile bacteria for enhancement of crop productivity.

1.2 Microbial Diversity

The term 'microorganism' encompasses an extensive and diverse assemblage of eukaryotic and prokaryotic organisms, such as bacteria, algae, protists and fungi which exhibiting with distinct morphological, ecological and physiological characteristics and satisfies the truly incredible microbial diversity on Earth. Evaluation of microbial diversity and community structure is an exclusive task because of taxonomic and methodological difficulties (Zak et al. 1994). Microbial diversity in soil ecosystem exceeds more than eukaryotic organisms. One gram of soil may harbor up to 10 billion microorganisms of possible thousands of different species (Roselló-Mora and Amann 2001). It describes complexity and variability at different levels of biological organization. It conceals genetic variability within taxons (species), and the number (richness) and relative abundance (evenness) of taxons and functional groups (guilds) in communities as stated by Torsvik et al. (2002). In soil ecosystem, microbial communities adapt microhabitat to live in, interacting and function in it. Interaction of microorganisms in a community can alter their community dynamics and size. Competitive interactions are thought to be a key factor controlling microbial community structure and diversity (Jha et al. 2010). Microorganisms inhabit with relative diversity even in extreme environments. To approach the study of microbial diversity, taxonomic and functional diversity are major concerns where, taxonomic diversity integrates different aspects of microbial diversity and provides a more complete picture with deeper understanding of microbial interactions in soil ecosystems (Torsvik et al. 2002). For instance, taxonomy of the bacteria studied by Misko and Germida (2002) accounted genera *Stenotrophomonas*, *Flavobacterium*,

Arthrobacter, *Stenotrophomonas*, *Pseudomonas* and *S. maltophilia* in rhizospheric soil. In such study, the rhizosphere, in general, has more prone with Gram-negative bacteria than Gram-positive and suggest bacterial populations were taxonomically diverse. On the other hand, functional diversity explores distinctions and similarities on the basis of functionality in their ecological niche or ecosystem.

1.2.1 Taxonomic Diversity

Taxonomic diversity considered both on phenotypic and genotypic level. The phenotypic diversity includes the morphological, physiological and biochemical characteristics and genotypic diversity include genetic material only. On both level, microorganisms are subjected to taxonomically identified and differentiated in comparison made with earlier known bacteria.

1.2.1.1 Phenotypic Diversity

The phenotypic approach in rectification of diversity with microbial community limits within culture dependent techniques. Phenotypic diversity considers the morphological, biochemical and physiological characteristics which are able to diversify the population of bacteria so, initially, to explore the divergence/similarity within the community of bacterial strain isolation and phenotypic characterization on morphological, physiological and biochemical traits. Torsvik et al. (1990) characterized the strain phenotypically using API20B test system based on 20 serial biochemical reactions. In such study, strains were subjected to cluster analysis which revealed 41 biotype with 80 % similarity among five dominant biotypes contain 43 % strains. Elucidation of phenotypic diversity was performed using ecological statistical measures particularly Shannon's and Simpson's Index. Mavingui et al. (1992) isolated rhizospheric and non-rhizospheric community of *Bacillus polymyxa* from wheat rhizosphere to decipher only phenotypic diversity based on morphological, API50CHB and API20B characterization and cluster analyzed by un-weighted pair group mean analysis (UPGMA) which indicated distribution of microbes in four groups at 93 % of similarity level. These studies confirm the existence of diversity even at strain level in bacteria from rhizosphere and non-rhizosphere soil. Berg et al. (2002) isolated 5854 bacteria from the rhizosphere of strawberry, potato, oilseed rape and bulk soil and screened of their antagonistic function against pathogenic fungi. Limited physiological characters viz., glucanolytic, chitinolytic and proteolytic activities were performed to determine rhizospheric bacterial diversity. Woyessa and Assefa (2011) characterized 66 isolates by several morphological, physiological and biochemical tests and identified to species level using API kit method. Numerical analysis of phenotypic data using UPGMA (NTSYSpc software version 2.1) was performed on the basis of carbohydrate fermentation profile. In the results, phenotypic data showed 50% average similarity of two major clusters represented both Gram-negative and Gram-positive isolates while the respective

groups form six and seven clusters at about 75% and 78.4% average similarity. González et al. (2013) reported phenotypic diversity in the population of 881 bacteria which include Gram-positive sporulated and Gram-positive non-sporulated bacteria that were statistically analyzed by hierarchical ascending classification (HAC) using parameters such as proximity type (similarity), agglomeration method (Jaccard), clustering method (medium link), and truncation level (0.03). It is interesting to note that the highest diversity of species was found in *Bacillus cereus*, *B. thuringiensis*, *B. anthracis*, *B. mycoides*, *B. megaterium*, and non-spore forming Gram-positive bacilli, *B. stearothermophilus* and *B. lentus*.

1.2.1.2 Genotypic Diversity

The term “Genotypic Diversity” is the paradigm of genetic divergence in microbial community. Genotypic diversity or genetic diversity is the lowest level, at which a population cannot evolve and adapt to environmental changes without genetic diversity. Genetic diversity is defined as the amount of genetic variability among individuals or population of species (Brown 1983). Phenotypic approach in rectification of diversity with microbial community limits within culture dependent techniques, although phenotypic characters govern by genetic material. Therefore, exploration of divergence implicit in genetic materials of bacterial population of certain ecosystem is important. The study of genetic diversity based on several molecular technique viz., Amplified Ribosomal DNA Restriction Analysis (ARDRA), Enterobacterial Repetitive Intergenic Consensus—Polymerase Chain Reaction (ERIC-PCR), Rapid Fragment Length Polymorphism (RFLP) and 16S rRNA or DNA sequencing. Genetic information of bacteria meets with much attention since last two decades. The use of molecular methods for study of genetic diversity primarily the sensitive and accurate PCR-based genotyping methods enables differentiation among closely related bacterial strains and the detection of higher rhizobial diversity than previously considered (Doignon-Bourcier et al. 2000; Tan et al. 2001). Laguerre et al. (1994) applied Restriction Fragment Length Polymorphism (RFLP) analysis of PCR-amplified 16S rDNA for identification of rhizobia. This technique has also been utilized by several workers for the identification of several novel species (Tan et al. 2001). Randomly Amplified Polymorphic DNA (RAPD) profiles have provided new tools for investigating genetic polymorphism (Bisht et al. 2013).

Genetic diversity within the rhizosphere has paid much attention by several scientific contributions (Upadhyay et al. 2009; de Salamone et al. 2010; Venieraki et al. 2011; Pisa et al. 2011; Saikia et al. 2011; Kim et al. 2011; Shiro et al. 2013; Dubey et al. 2013). Attempts have been made to explore the genetic divergence within the bacterial population of the rhizospheric ecology. Kumar et al. (2006) used such tools to study genetic diversity of isolates from *Mucuna pruriens* rhizosphere. Tian et al. (2009) identified 28 ARDRA pattern among the 299 siderophore producing bacterial isolates of tobacco rhizosphere and found 14 different genera from ARDRA pattern namely α -Proteobacteria, β -Proteobacteria and γ -Proteobacteria, *Sphingobacteria*, *Bacilli* and *Actinobacteria*. Especially γ -Proteobacteria consisting of genera *Pseudomonas*, *Enterobacter*, *Serratia*, *Pantoea*, *Erwinia* and *Stenotroph-*

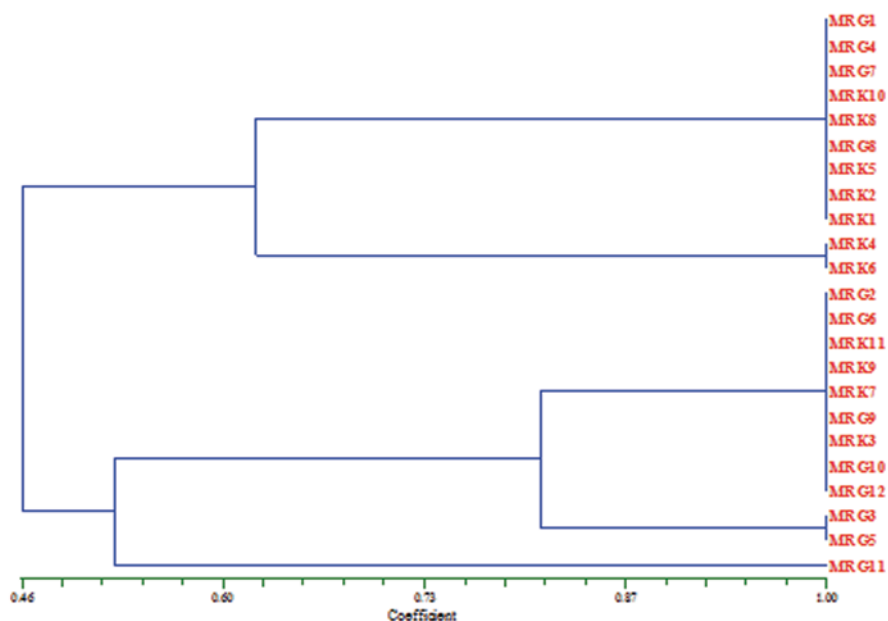


Fig. 1.1 Dendrogram based on ERIC-PCR of rhizobial isolates showing genetic divergence using Jaccard's Coefficient and UPGMA cluster method using NTSYS Software Ver. 2.1

omonas. Recently, from author's lab, Dubey et al. (2010) investigated the diversity of fast growing rhizobia of pigeon pea nodules based on variation in partial sequences of the 16S rRNA gene and RFLP. Susilowati et al. (2010) studied the genetic diversity of the antifungal *Pseudomonas* sp. using Amplified 16S rDNA Restriction Analysis (ARDRA) and 16S rRNA gene sequences analysis and determine the clustering analysis of the amplified rDNA distinguished *Pseudomonas* sp. into seven groups. The sequences of 16S rRNA gene confirmed that the isolates similar to *Pseudomonas* sp. and the phylogenetic tree constructed formed four clusters. Findings suggested that antifungal *Pseudomonas* sp. were present with significant genetic diversity in existing species in the rhizosphere of soybean plant. Recently, Solanki et al. (2012) assessed genetic diversity within 108 *Bacillus* isolates using 16S rDNA, BOX and ERIC-PCR. Kadyan et al. (2013) studied the genetic diversity of 52 functional plant growth promoting rhizobacteria PGPR using 16S rDNA sequencing and were found similarity with in six different genera of aerobic endospore forming bacteria (AEFB) i.e., *Bacillus*, *Brevibacillus*, *Lysinsibacillus*, *Paenibacillus*, *Terribacillus* and *Jeotgalibacillus*. In our recent study in which several rhizobia proceeded for ERIC-PCR tool of molecular analysis, based polymorphic band pattern were obtained that confers the genetic diversity of rhizobia with different gene structure. Based on distinct band pattern UPGMA cluster analysis was performed and dendrogram was constructed, which satisfies significant diversity within rhizobial isolates (Fig. 1.1).

1.2.2 *Functional Diversity*

Biological diversity is puzzled with co-existence of species but, how they are functional in their ecology and what is the degree of functionality of different genera in particular is studied under the term of functional diversity. Functional diversity is the diversity of species trait in an ecosystem. Concerning the bacterial diversity of rhizosphere pursuit functionality on account of their plant growth promoting (PGP) activity on that basis they are relatively diverse for rhizospheric importance (Khan et al. 2009). Functional diversity influence ecosystem dynamics, stability, productivity, nutrient balance and other aspects of ecosystem functioning (Tilman 2001). Functional diversity in rhizosphere is in the lime light of study because, the rhizosphere is a narrow zone of soil immediately surrounding to the plant roots and this zone consists high biological activity in term of nutrient cycling or management and habitan PGPR with symbiotic and non-symbiotic interactions. Embedded microbial community of rhizosphere possesses functional diversity which substantiates agricultural production (Tilak et al. 2005). Further, the diversity of bacterial communities present in the rhizosphere heavily influences soil and plant quality with ecosystemic sustainability (Trivedi et al. 2011).

Functional diversity measures the distribution and the range of what organisms perform to that of microbial communities and ecosystems, and thus considers the complementarity and redundancy of co-occurring species (Díaz and Cabido 2001; Petchey and Gaston 2006). Functional diversity is usually rumored for ecosystem productivity and vulnerability by its member's diversity (Tilman et al. 1997; Hulot et al. 2000; Díaz and Cabido 2001; Heemsbergen et al. 2004). Functional diversity studies multi-dimensional cloud of species trait and each trait representing an individual or a species e.g., Phosphate Solubilizing Bacteria (PSB), whereas, phosphate solubilization is a function of rhizospheric microorganism to alleviate the nutritional requirement in soil, on further aid in plant growth promotion. Such groups of selective individuals are considered as functionally diverse and utilize to improve agricultural production (Maheshwari 2011, 2012, 2013). Schroth (1981) termed these beneficial rhizobacteria as plant growth-promoting rhizobacteria (PGPR). Plant growth-promoting rhizobacteria (PGPR) enhance plant growth by a wide variety of mechanisms like biological nitrogen fixation, phytohormone production, phosphate solubilization, siderophore production, 1-aminocyclopropane-1-carboxylate deaminase production (ACC), exhibiting antifungal activity, production of volatile organic compounds (VOCs) promoting beneficial plant-microbe symbiosis, interference with pathogen toxin production etc. The functionality of PGPR in agriculture is bit by bit increased with its diversity. PGPR are more diverse within two broad categories in accordance with their mode of association with the plant root cells; extracellular PGPR (ePGPR) and intracellular PGPR (iPGPR) as stated by Martinez-Viveros et al. (2010). These two classes only diverse on the basis of their ecological niche, even the habitat is same for both i.e., rhizosphere.

Functional diversity in the rhizosphere is considered on the mechanistic behavior of microbial guilds which ultimately promotes the plant growth. More than a decade ago, Hellriegel and Wilfarth (1888) investigated the rhizosphere root colonization in grasses and legumes and suggested the functionality of soil bacteria to convert atmospheric

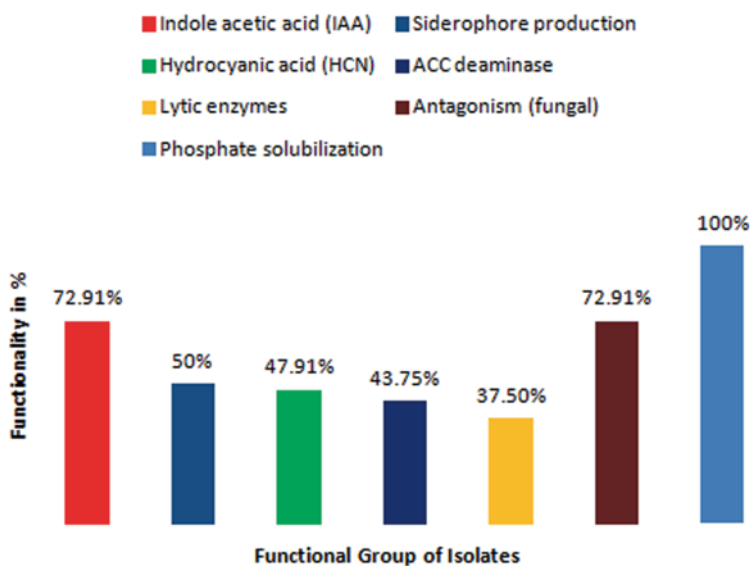


Fig. 1.2 Functionality of *Bacillus* isolates screened on PGP attributes in percentage (e.g., out of 48 isolate, 72.91 % n=35)

N₂ into plant usable forms. Saikia et al. (2011) studied functional diversity of fluorescent *Pseudomonas* from the tea rhizosphere as they are functional as biocontrol agent against several phytopathogenic fungi. Kadyan et al. (2013) studied functional diversity on the basis of PGP trait in aerobic endospore forming bacteria (AEFB) isolated from *Phyllanthus amarus* rhizosphere and found 92.30% strains functional to produce IAA, 86.53% for phosphate solubilization, 44.23% strains for siderophore production, 42.30% strain for chitinase production, 21.15% strains for 1-amino cyclopropane-1-carboxylate (ACC) deaminase production and 46.15% strains are functional for antagonistic activity against common fungal pathogen of the plant. This study reflects the functional diversity among the total cultivable population of *P. amarus* rhizosphere. In our recent study on *Bacillus* isolated from rhizospheric soil, we evaluated for functionality on the basis of PGP attributes, in which bacteria reflect relative diversity as they show 28.57–85.71 % functionality. The population of isolated *bacilli* shows 100% functionality in phosphate solubilization, 72.91% IAA production, 72.91% antagonism, 50% siderophore production, 47.91% HCN production, 43.75% ACC deaminase production and 37.50% lytic enzyme production (Fig. 1.2).

1.3 Levels of Microbial Diversity

Biologists most often define biodiversity as the “totality of genes, species, and ecosystems of a region” (Larsson 2001). Hawksworth (1996) accepted definition from United Nation Earth Summit (Quarrie 1992) of biological diversity that “the biological

diversity means the variability among living organisms from all sources including, inter alia, terrestrial, marine and other aquatic systems and the ecological complexes of which they are part of; this includes diversity within species, between species and of ecosystem.” Based on this definition, which describe the three traditional levels of biodiversity i.e., species diversity, genetic diversity and ecological diversity, similarly also spreads on microbial diversity (Hill et al. 2003; Kirk et al. 2004; Fierer et al. 2007).

1.3.1 *Species Diversity*

Species richness is defined as the number of species in a sample unit or other specified area. According to Whittaker (1972) species diversity is the measure of various species represents a collection of individuals or a community which is weighted by abundance of different species in a population or ecosystem. It includes species richness in term of number of species in a community. The relative abundance of species is also important to consider how common and rare species is relative to other species in a community. Relative abundance of rare and common species is called species evenness. Evenness is simply a measure of how species are similar in their abundances (Lloyd and Gherlardi 1964). Whittaker (1972) defined three levels of diversity; Alpha (α) diversity, Beta (β) diversity and Gamma (γ) diversity. According to which total species diversity is determined by mean species diversity in a habitat (α diversity) and differentiation among species of those habitats (β diversity) together with the term of γ diversity. According to Colwell (2009), α -diversity expressed with the number of species of a particular ecosystem (i.e., species richness), during a study evaluation of species diversity on comparative scale is to find unique species in both/many ecosystem (pair wise in case of many ecosystem) considers as β -diversity. On the other hand, γ -diversity is an overall diversity for the different ecosystems within a region and concisely defined as “geographic-scale species diversity.” Mayden (1997) defines what a species constitute under the term of “species concept.” Long back, Regan (1926) further explained in the term of “morphological species concept” and described as “a species is a community or a number of related communities, whose distinctive morphological characters are, in the opinion of competent systematist, sufficiently definite to entitle it, or them, to a specific name.” Cracraft (1983) defined the “phylogenetic species concept” as “is the smallest diagnosable cluster of individual organism (that is, the cluster of organisms is identifiably distinct from other clusters) within which there is a parental pattern of ancestry and descent.” This concept within the new imperative findings produced a unified concept of species, categorizing hierarchical and taxonomic ranks of biological organization (de Queiroz 2007).

1.3.2 *Genetic Diversity*

Genetic level of diversity is defined on variable genetic characters for genetic make-up of a species. In simpler context of genetic diversity is the divergence of species

in reference to its characters governs by DNA/RNA or both genetic materials. A genetic variation shapes and defines divergence among individuals, populations, subspecies, species, and strains ultimately at the kingdoms of life on earth. Existence of variability in genetics of individuals of a population is important for the determination of diversity as it enables certain population of microorganism to adapt the environment and therefore, they become more prone against environmental harness (Sousa et al. 2011). Genetic variation among individuals within a population has long been recognized as the starting block for adaptation and evolution among microorganisms as well as among other organisms (Morris et al. 2002).

Genetic diversity not only applies for the species and strains at the lowest rank of taxonomy, even it also applies on domain, the higher rank of taxonomy. Woese et al. (1990) using phylogenies based on ribosomal RNA sequence proposed natural classification system of organisms in three domains, as bacteria, archaea and eukarya. This classification is based on divergence in genetic material of most conserved sequences of 16S rRNA gene in the bacteria and 23S rRNA gene in other two domains i.e., archaea and eukarya. Genetic diversity is much higher in domain Bacteria and Archaea than that of the domain Eukarya. During the last decade, assessment of diversity within natural rhizobial populations in various regions of the world has received considerable attention (Ando and Yokoyama 1999; Chen et al. 2000; Kumar et al. 2006; Selvakumar et al. 2007). Wolde-Meskel et al. (2005) studied genetic diversity of rhizobial strains isolated from root nodules of 18 agroforestry legumes of Ethiopia by using PCR-RFLP of 16S rRNA gene, 23S rRNA gene, the Internal Transcribed Spacer (ITS) region and 16S rRNA gene partial sequence (800 and 1350 bp) analysis. Further, Liu et al. (1997) developed quantitative molecular technique for rapid analysis of microbial diversity in which PCR technique was employed for to amplify specific region of 16S rRNA gene, further digested with restriction enzymes and fluorescent labeled terminal restriction enzyme, measured using automated DNA sequencer. Terminal Restriction Fragment Length Polymorphisms (T-RFLP) revealed high bacterial diversity. Genetic diversity is evaluated by several other advance molecular methods e.g., ADRDA, ERIC-PCR which enables the differentiation between species and strain of the same species (Pinto et al. 2007; Wang et al. 2009). RFLP-PCR associated with ribosomal genes is another useful technique that has been used for phylogenetic studies, as it can discriminate rhizobial species and generally exhibits good agreement with partial or complete gene sequencing data (Laguerre et al. 1996; Vinuesa et al. 1998; Diouf et al. 2000; Andrade et al. 2002; Fernandes et al. 2003; Pandey et al. 2004; Moschetti et al. 2005; Kumar et al. 2006). Pisa et al. (2011) explored diversity of 16S rRNA gene from bacteria of sugarcane rhizosphere. In our recent study, diversity of rhizobial community was revealed by ERIC-PCR technique among 23 isolates and putative strains were identified based on 16S rRNA gene sequences using on-line software of sequence alignment viz., BLASTn and query sequence of bacterial strain based on which strain is identified as *Rhizobium leguminosarum* with due result of similarity with reference strains in sequence alignment. Phylogenetic tree construct using phylogenetic tree construction software, viz., MEGA 6.0 (Tamura et al. 2013) that permit access of particular strain to place it in its taxonomy.

1.3.3 Ecological Diversity

Ecological diversity is defined as the variety and abundance of species in different habitats and communities. Ecological diversity means the diversity on the ecosystem level. In other words “ecological diversity refers the variety of ecosystems present in a biosphere aids variety of species and their ecological processes under physical, chemical and biological factors. Under concern of ecological diversity, rhizosphere is microbial community prone ecology, where micro-organisms lives with plant root in several interactions like symbiosis, parasitism, synergism etc. Plant microbe interactions are controlled by the transfer of nutrients from plant roots in the rhizosphere and populations of bacteria have functional roles within communities that permit their survival. In rhizosphere frequently interactions with each other between different bacterial species have been demonstrated in different ecosystems (Pandey and Maheshwari 2007; Maheshwari 2012).

In the term of ecological diversity, variation in community structure, complexity of interactions, number of trophic level, and number of guilds are included. Yang and Crowley (2000) showed that the microbial communities associated with the different root locations produced many common 16S rDNA bands on marker studies but these communities could be distinguished by using corresponding statistical analysis. However, bacterial communities in the rhizosphere are substantially different from different root zones such as root cap, apical meristem, procambium, ground meristem, protoderm, and root hairs. A rhizosphere community may be altered by changes in root exudate composition leads the changes in nutritional status. On other hand, Doornbos et al. (2011) concerned over the effects of plant genotype on rhizosphere bacterial community structure and found no relationship to plant defense because chemical activation of induced systemic resistance (ISR) or systemic acquired resistance (SAR) had no significant effects on density and structure of the rhizospheric community resultant none effect on the resident soil bacteria. Most common bacteria in the ecology of rhizosphere includes *Actinoplanes*, *Agrobacterium*, *Alcaligenes*, *Amorphosporangium*, *Arthrobacter*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Paenibacillus*, *Burkholderia*, *Cellulomonas*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Gluconacetobacter*, *Microbacterium*, *Micromonospora*, *Pseudomonas*, *Rhodopseudomonas*, *Rhizobia*, *Serratia*, *Streptomyces*, *Xanthomonas*, etc., as stated by several workers (Kloepper et al. 1989; Tang 1994; Okon and Labandera-Gonzalez 1994; Glick et al. 1999; Mayak et al. 2001; Tahmatsidou et al. 2006; Aslantas et al. 2007; Lee et al. 2008; Pedraza et al. 2010). In rhizospheric ecology complexity in interaction occurs in both managed and neutral ways. The plant-associated bacteria migrate to the rhizosphere to get rid of colonization. The colonization processes is important to better predict how bacteria interact with plants and whether they are likely to establish themselves in the plant environment (Compant et al. 2010). Ibekwe et al. (2010) analyzed the bacterial diversity under the effects of abiotic factors such as salinity, boron, and pH were more important to maintain the rhizosphere bacterial population with decreasing impacts with plant growth. The effects were significant in the rhizosphere and on species richness and diversity. Recently, Eissfeller et al. (2013) found that rhizospheric community variably and in particular have higher trophic levels rely on carbon and originating substances.

1.4 Measuring Microbial Diversity

Measurement of microbial diversity is a daunting task for ecologist. Traditionally there are three levels at which diversity has been described. In fact it uses genetic diversity as a basis for valuing both species diversity (for their relative richness in different genes) and ecosystem diversity (for the relative richness in the different processes to which the genes ultimately contribute). Measuring diversity on an ecosystem level is assumed to be a better way of observing at the shape of the entire system, rather than the particular species. From the number of ecological diversity measures, some are considered suitable to measure highly diverse bacterial communities as in particular, species richness and evenness/dominance indices, species abundance models. Earlier, Magurran (1988) grouped diversity measurement into three categories: species richness indices, evenness and dominance indices and species abundance model. Based on species richness indices, it is simple to count species by Margalf's and Menhinick's diversity index for which Clifford and Stephenson (1975) attempted to compensate richness in the term of number of species observed divided by total number of individual in sample. Whereas, Menhinick's index has slight alternation in calculation as it uses root of total number of individual as dividend. Evenness and dominance indices quantifies how equal the species in the ecosystem markedly analyzed by Simpson's Index, Shannon-Weiner's Index, Berger-Parker's Index and Lloyd-Ghelardi Index (Hill et al. 2003; Atlas and Bartha 1998). Whereas, the Simpson's index gives the probability of any two individuals drawn at random from any infinity large community belongs to the same species. On the other hand, Simpson's diversity measures emphasizes on the dominance as opposed to the richness, component of diversity (Magurran 2006). Species abundance model are more sophisticated tool to investigate diversity because they impound to elucidate the distribution of abundance in a population. Using statistical model; log series, log normal (Preston 1948) and biological niche based model (Tokeshi 1999) describe mathematical relationship between number of species and number of individuals to establish abundance. The era of molecular microbial ecology has uncovered microbial diversity, which is based on rRNA and rDNA analysis (Torsvik and Overeas 2002).

1.5 Rhizosphere Implicit Microbial Diversity

The rhizosphere is the volume of soil around the root system of plants harboring diversity of microorganisms that interact with plant in virtue of shelter and nutritional requirement and significantly influence plant growth. Rhizospheric microbial population provision for plant in term of growth promotion is recognizes as plant growth promoting rhizobacteria (PGPR). Relationship between microorganisms and plants confers the availability of nitrogen along with other nutritional requirement viz., nitrogen, phosphorus, zinc, potassium etc. Plant roots offer a niche to proliferation of soil bacteria and thrive their life under influence of root exudates and lysates. Root exudation is part of the rhizodeposition process, which is a major source of

soil organic carbon released by plant roots (Hütsch et al. 2002; Nguyen 2003). Plant root exudates directly affect microbial populations within the rhizosphere (Whipps 2001; Buyer et al. 2002; Welbaum et al. 2004; Morgan et al. 2005; Broeckling et al. 2008) by providing growth substrates such as sugars, amino acids, organic acids, fatty acids, nucleotides, sterols, vitamins and other compounds that influence the growth of bacteria and fungi. Root exudates mediate both positive and negative interactions in the rhizosphere. The positive interactions include symbiotic associations with beneficial microbes, such as mycorrhizae, rhizobia and PGPR, whereas, negative interactions include association with parasitic plants, pathogenic microbes and invertebrate herbivores. Bacteria, in turn of nutritional availability in the rhizospheric niche employed in growth promotion of plant by increasing soil fertility aiding nutritional richness in soil.

PGPR originally defined as root colonizing bacteria (rhizobacteria) that provide either growth promotion or biological control of plant diseases. The mechanism of PGP is categorized into the direct and indirect plant growth-promoting mechanisms (Glick et al. 1995). A variety of bacteria have been used as soil inoculants intended to improve the supply of nutrients to crop plants. At this juncture, the significance of rhizobia cannot be ruled out. Taxonomy of rhizobia is diverse parallel to the evolution of molecular biology and polyphasic approach that leads continuous increase in the number Rhizobial genera. Under more diverse taxonomy, rhizobia belongs to α - and β -proteobacteria and consist of 95 species within 13 genera, viz., *Azorhizobium*, *Bradyrhizobium*, *Burkholderia*, *Cupriavidus*, *Devosia*, *Ensifer*, *Herbaspirillum*, *Mesorhizobium*, *Methylobacterium*, *Ochrobacterium*, *Phyllobacterium*, *Shinella* and *Rhizobium* (Maheshwari et al. 2013). Similarly, as stated earlier, the ePGPRs may exist in the rhizosphere, on the rhizoplane or in the spaces between the cells of root cortex. The bacterial genera such as *Agrobacterium*, *Arthrobacter*, *Azotobacter*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Caulobacter*, *Chromobacterium*, *Erwinia*, *Flavobacterium*, *Micrococcus*, *Pseudomonas* and *Serratia* belongs to ePGPR (Gray and Smith 2005; Bhattacharyya and Jha 2012). Some other bacteria responsible for plant growth promotion are *Azoarcus* sp., *Burkholderia* sp., *Glucanacetobacter diazotrophicus*, *Herbaspirillum* sp., *Azotobacter* sp., and *Paenibacillus polymyxa* (Vessey 2003). *Azoarcus* has gained much attention and consideration by virtue of its rich genetic and metabolic diversity and competitive advantages in a carbon-rich, nitrogen-poor environment (Reinhold-Hurek and Hurek 2000).

Current advancement in exploration of facts of information on rhizosphere diversity along with PGPR and their functional ability and mechanism to facilitate plant growth promotion corroborated their application as a reliable component in the management of sustainable agricultural system. Our understanding on microbial diversity, community dynamics in comparison to plant, animal diversity is less studied on account of soil type, plant type or stage of plant development (McSpadden 2004; Duineveld et al. 1998). The mystery behind it is limited attempts have made earlier to account unravel wealth of microbial diversity embedded in rhizosphere; it probably due to unavailability of appropriate methodology for research community. In the context to improve methodology to study rhizosphere implicit diversity several techniques are recently incorporated. Genetic diversity is studied by several

advance molecular methods e.g., ADRDA, ERIC-PCR which enables the differentiation between species and strain of the same species (Pinto et al. 2007; Wang et al. 2009). RFLP-PCR associated with ribosomal genes is another useful technique that has been used for phylogenetic studies, as it can discriminate rhizobial species and generally exhibits good agreement with partial or complete gene sequencing data (Laguerre et al. 1996; Vinuesa et al. 1998; Doignon-Bourcier et al. 2000; Mostasso et al. 2002; Grange and Hungria 2004; Kumar et al. 2006). El-Akhal et al. (2009) studied local origin of rhizobial strain on the basis of 16S rRNA analysis. PCR based RFLP an analysis of 16S rDNA has been evidenced as rapid and reliable methods for grouping new isolates at species level (Laguerre et al. 1994). Further it was noted that genetic diversity of rhizobial populations often studied to characterize more effective or better adapted strains for particular applications (McInnes et al. 2004). RFLP of PCR-amplified genes coding for 16S rRNA (ARDRA) is common method for the genotypic classification and relationship of *Rhizobium* and *Ensifer* (Jarabo-Lorenzo et al. 2000; Wang et al. 2009; Shamseldin et al. 2009; Mahdhi et al. 2012) and in certain cases of fluorescent pseudomonads (Ramezanpour 2009; Saikia et al. 2011). The ERIC-PCR profiles have been used to analyze genetic similarity among isolates. Repetitive primers ERIC (de Bruijn 1992) has shown high sensitivity in detecting the diversity of tropical rhizobia at the strain level, although it does not cluster strains at the species or genus level (Chen et al. 2000; Ferreira and Hungria 2002; Galli-Terasawa et al. 2003; Grange and Hungria 2004; Kaschuk et al. 2006).

Available literature revealed limited work to signify the diversity of rhizospheric bacteria probably due to lack of appropriate technique and suitable methods. Reva et al. (2002) studied the diversity of endophytic AEFB present in the inner tissue of healthy crops and proved that *Bacillus licheniformis*, *B. megaterium*, *B. pumilus* and *B. subtilis* are found more abundant as root colonizer. Screening of *bacilli* on PGP attributes to determine the ecological and genetic diversity earlier also studied by Johri et al. (2003).

Genetic diversity and plant growth promoting (PGP) activities of nitrogen fixing *bacilli* have been evaluated by Beneduzi et al. (2008). Calvo et al. (2009) noted the ecological bacterial diversity and its consequences for functionality in the rhizosphere. Variations in genetic structure lead the ecological diversity within the species of *Bacillus* (Kumar et al. 2012). Recently, diversity of *bacilli* on physiological cultural characters from Lonar Lake, India is described by Tambekar and Dhundale (2012). Earlier, Wang et al. (1999) has explored diversity of rhizobia associated with *Amorpha fruticosa* isolated from Chinese soil and proposed for novel species *Mesorhizobium amorphae* based upon their distinct phylogenetic position. Anyango et al. (1995) reported diversity in root nodulating rhizobia in *Phaseolus vulgaris* L. in two soil samples of different pH. Another study on genetic diversity of PGPR *Pseudomonas* associative with different crop plants cultivated in saline farming is reported by Prabavathy et al. (2011). The genetic diversity of PGPR fluorescent pseudomonads associated with the sugarcane (*Saccharum officinarum* L.) rhizosphere was analyzed using 16S rRNA gene sequencing and *Pseudomonas plecoglossicida*, *P. fluorescens*, *P. libaniensis*, and *P. aeruginosa* dominantly

reported. Differentiation of isolates was achieved through different genomic DNA fingerprinting techniques, including randomly amplified polymorphic DNA (RAPD), amplified ribosomal DNA restriction analysis (ARDRA), repetitive extragenic palindromic (REP), enterobacterial repetitive intergenic consensus (ERIC), and bacterial repetitive BOX elements (BOX) analyses (Rameshkumar et al. 2012).

1.6 Microbial Diversity in Agricultural Benefits

Microbial diversity of rhizosphere bears the variety of microorganisms in the benefit of plant ecosystem. Diverse bacterial community in the rhizosphere are involved in various mechanisms to promote plant growth such as nitrogen fixation, plant growth hormone production, phosphate solubilization, siderophore production, HCN production, ACC deaminase production and bio-control of several phytopathogenic fungi. PGPR therefore, also aimed to investigate in relation to available plant essential nutrient in soil (Freitas et al. 2007). Significant increase in growth and yield of important crops in response to inoculation with PGPR have been repeatedly reported (Kloepper et al. 1980; Chen et al. 1994; Zhang et al. 1996; Amara and Dahdoh 1997; Pan et al. 1999; Gupta et al. 2000; Mariano and Kloepper 2000; Asghar et al. 2002; Vessey 2003; Gray and Smith 2005; Silva et al. 2006; de Araujo 2008). On the other hand, bacteria in the genera *Bacillus*, *Streptomyces*, *Pseudomonas*, *Burkholderia*, and *Agrobacterium* are the biological control agents suppress plant disease through production of antibiotics, enzymes and/or siderophores and ISR (Maheshwari et al. 2013; Uppal et al. 2008).

In the study of indigenous bacteria which were isolated from the root nodule and rhizospheric soil respectively and identified as *Rhizobium* and *Pseudomonas*. Further strains were screened for PGP attributes and used to predicate application potential in term of productivity enhancement under field trial. In which productivity of *Macrotyloma uniflorum* L. was reported to increase with the use of *Rhizobium* and *Pseudomonas* in field trials (Table 1.1).

Diverse genera of PGPR provide effective and eco-friendly alternatives to replenish agrochemicals, as they have been established to improve the yield and growth of crop plants (Vessey 2003). Bio-formulations with potential PGP strains/consortia to apply in the field assure the significance of PGPR in agriculture (Date 2001). Application of spore forming *Bacillus* and *Paenibacillus* species leads to increase in growth and yield of different crops (Vessey and Buss 2002; Nelson 2004).

1.7 Conclusion

In the broad sense, microbial diversity is prominent aspects of recent era. Under the quest of exploration of microbial diversity that conceptualize the understanding of accessory term of microbial diversity proved significant. Microbial diversity

Table 1.1 Field data depicting yield parameters of *M. uniflorum* (120 DAS) using (*Rhizobium* spp. (MRG6 and MRK10) and *Pseudomonas* spp. (FPG3 and FPK5; unpublished data from author's lab)

Treatment	No. of pods/ plant	Grain yield (kg/hectare)	Biological yield (kg/ hectare)	Harvest index	% rise over control
MRG6	18.333**	1189*	4603***	25.83	32.2
MRK10	16.667**	1073*	4400***	24.30	19.3
FPG3	19.333**	1247*	4700***	26.53	38.7
FPK5	17.667**	1131*	4485***	25.20	25.8
Control	14.333	899	3806***	23.62	
SEM	0.316				
CD at 1 %	1.497				
CD at 5 %	1.030				

Values are mean of ten replicates

* significant at 0.01 level of analysis of variance (ANOVA);

** significant at 0.01 level of LSD as compared to control;

*** significant at 0.05 level of LSD as compared to control;

^{ns} not significant at 0.05 level of LSD as compared to control

encompass as a whole meaning “variety” and divergence in more widely distributed microorganism holds significant diversity on taxonomic, functional and ecological level. During the exploration of the recent scenario of bacterial diversity several research advancement to assess the diversity on traditional levels: species diversity, genetic diversity and ecological diversity is interesting and unlocks new horizon of research in that. Among the diverse community of rhizospheric bacteria elite potential bacteria are in much concern as bio-inoculant for sustainable agriculture. On the virtue of this respect, bacterial diversity of rhizosphere is too meaningful to use for agriculture welfare. Sequentially diverse community of rhizobacteria part in the production and productivity of severe crops, leads the economic development.

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Chapter 2

Diversity Utility and Potential of Actinobacteria in the Agro-Ecosystem

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Abstract Actinobacteria formerly referred to as Actinomycetes are Gram-positive saprophytic bacteria, with widespread distribution in nature. They occur in the terrestrial and aquatic environments, and play a dominant role in natural geochemical cycles. Mankind's interest in Actinobacteria, originated in the early nineteenth century, primarily due to their abilities to decompose organic matter and produce antibiotics. Amongst Actinobacteria, the genus *Streptomyces* has received widespread attention due to its ability to produce biologically active compounds that have been widely exploited against infectious agents. But emerging trends in microbial architecture and taxonomy have led to the re-defining of this group of microbes, and have led to the inclusion of several known and novel bacterial genera and species, within the Phylum Actinobacteria. These are widespread in the agro-environment, especially in the rhizosphere, where they produce a wide range of biologically active metabolites and influence plant development in a myriad fashion. We attempt to capture the existing information on the diversity and utility of Actinobacteria in the agro-environment, and the interventions that are required in the future in order to fully exploit this class of microbes, for the benefit of mankind.

2.1 Actinobacteria—An Introduction

Actinobacteria formerly referred to as Actinomycetes or Ray fungi are Gram-positive, saprophytic bacteria, with widespread distribution in nature. The Phylum Actinobacteria represents one of the largest taxonomic units, among the currently recognized major lineages within the domain *Bacteria* (Stackebrandt et al. 1997). Actinobacteria are Gram-positive with a high G+C content in their DNA. The G+C content ranges from 51 % in some *Corynebacteria* to more than 70 % in *Streptomyces* and *Frankia*. An exception to this is the genome of the obligate pathogen *Tropheryma whipplei*, with less than 50 % G+C. Actinobacterial morphologies range from coccoid (*Micrococcus*) or rod-coccoid (e.g., *Arthrobacter*) to fragmenting hyphal forms (e.g., *Nocardia* sp.) or permanent and highly differentiated branched

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Table 2.1 A schematic representation of the diversity within Phylum Actinobacteria as laid out in the Bergeys Manual of Systematic Bacteriology (2nd edition)—Volume 5. (Source: Abridged from Ludwig et al. (2012b)).

Phylum: Actinobacteria					
Classes (6)					
<i>Actinobacteria</i>	<i>Acidimicrobiia</i>	<i>Coriobacteriia</i>	<i>Nitriliruptoria</i>	<i>Rubrobacteria</i>	<i>Thermoleophilina</i>
Orders (15 + 1 ^a)	Order (1)	Order (1)	Orders (2)	Order (1)	Order (2)
Families (43)	Families (2)	Family (1)	Families (2)	Family (1)	Families (4)
Genera (128)	Genera (5)	Genera (13)	Genera (2)	Genus (1)	Genera (4)

The figures in parentheses represent the numerical value of the taxonomical unit

The numerical value includes taxa that appear in the approved list, validly published and taxa that are not validly published as on 01-01-2008

^a Order—*Incertae sedis* (uncertain placement)

mycelium e.g., *Streptomyces* sp. (Atlas 1997). Some unusual developmental features displayed by some actinobacterial genera, include the production of sporulating aerial mycelium which is a common feature of the genus *Streptomyces* or the persistent non-replicating state exhibited by certain mycobacteria. While most members of the Actinobacteria are aerobic, few can grow under anaerobic conditions. Though widely known as soil dwellers, Actinobacteria are also present in a wide variety of aquatic and marine environments (Asquith et al. 2013).

The metabolic versatility of Actinobacteria stems from their ability to secrete diverse metabolites ranging from extracellular enzymes to antibiotics. Notable among these metabolites are the antibiotics (Lechevalier and Lechevalier 1967), by virtue of which the Actinobacteria tend to occupy a coveted position in the pharmaceutical industry. Actinobacteria also exhibit diverse lifestyles in nature, wherein they occur as pathogens (e.g., *Mycobacterium* sp., *Nocardia* sp., *Tropheryma* sp., *Corynebacterium* sp., and *Propionibacterium* sp.), soil inhabitants (*Streptomyces* sp.), plant commensals (*Leifsonia* sp.), nitrogen-fixing symbionts (*Frankia*), and gastrointestinal tract inhabitants (*Bifidobacterium* sp.) (Ventura et al. 2007). Considering their morphological, physiological and functional utility, Actinobacteria have been rightly assigned a distinct position amongst bacteria. The diversity and importance of the Actinobacteria can be gauged from the fact that the second edition of the Bergey's Manual of Determinative Bacteriology has dedicated an entire volume for the Phylum Actinobacteria. This volume comprises of two parts, and contains descriptions of Actinobacteria based on their phylogenetic affinities (Ludwig et al. 2012b). The Phylum Actinobacteria is now divided into six classes, which contain several orders and families (Ludwig et al. 2012a). A brief schematic representation of the taxonomic distribution of Phylum Actinobacteria is given in Table 2.1.

Subsequent to the taxonomic delineation of Actinobacteria, as outlined in the Bergey's manual, many new genera and species continue to be validly published. This chapter is intended to throw light on the diversity, potential and utility of Actinobacteria, within the agro-ecosystem, where they play important roles in nutrient cycling, disease suppression and plant growth promotion, besides causing diseases in a number of cultivated plants.

2.2 Diversity and Utility of Actinobacteria in the Agro-Ecosystem

Within agroecosystems, actinobacteria have several utilities, which are described in greater detail in the ensuing sections.

2.2.1 *Actinobacteria as Plant Disease Suppressors and Sources of Agro-Active Antibiotics*

Actinobacteria have long gained significance in the agro-environment due to their ability to produce a wide range of antibiotic molecules that suppress the growth and development of a wide range of soil dwelling plant pathogens. It is estimated that as many as three-quarters of all *Streptomyces* species are capable of antibiotic production (Alexander 1977). This can be gauged from the fact that nearly 1000 secondary metabolites were discovered from Actinobacterial sources during the period 1988–1992, with the genus *Streptomyces* being single most contributor to this (Tanaka and Omura 1993). Apart from antibiotic production, several Actinobacteria have the ability to colonize plant surfaces and thereby exclude plant pathogens. Parasitization of the fungal pathogens is another mode of plant disease suppression by Actinobacteria (Yuan and Crawford 1995). Some Actinobacteria impede the growth of plant pathogenic organisms by the production of high levels of extracellular lytic enzymes such as the chitinases and the glucanases (Mahadevan and Crawford 1997; El-Tarabily 2006). The degradation of signal molecules involved in the pathogenesis quorum-sensing (Uroz et al. 2003), and the induction of plant resistance mechanisms (Shimizu et al. 2005; Conn et al. 2008) are also commonly encountered amongst Actinobacteria.

Most path breaking discoveries of agro-active antibiotic molecules originated from Japan, during the later part of the last century and many of the compounds are still in use. Some of the early important agro-active antibiotic molecules that were isolated from Actinobacteria, and have been put to commercial use are described herein. Kasugamycin a bactericidal and fungicidal metabolite obtained from *Streptomyces kasugaensis* (Umezawa et al. 1965) inhibits protein synthesis in microorganisms but not in mammals. The Hokko Chemical Industries, Japan has developed systemically active kasugamycin for control of rice blast caused by the fungus *Pyricularia oryzae* and bacterial diseases caused by *Pseudomonas* in several crops. Similarly, Polyoxin B and D isolated as metabolites of *Streptomyces cacaoi* var. *asoensis* by Isono et al. (1965), primarily interfere with the fungal cell wall synthesis by specifically inhibiting the enzyme chitin synthase (Endo and Misato 1969). Polyoxin B was deployed against a wide range of fungal pathogens in fruits, vegetables and ornamental crops. It is also used to control rice sheath blight caused by *Rhizoctonia solani*. The Validamycin family of antibiotics were discovered in 1968. Validamycin A is a pro-drug which is converted within the fungal cell to validoxylamine A, an extremely strong inhibitor of trehalase (Kameda et al. 1987). This mode of action gives, Validamycin A, a toxicological

edge since vertebrates do not depend on the hydrolysis of the disaccharide trehalose for their metabolism. The antifungal metabolite Mildiomycin obtained from *Streptovercillium rimofaciens* (Iwasa et al. 1978), is highly active against several powdery mildews on various crops (Harada and Kishi 1978). This inhibits fungal protein biosynthesis and is fairly safe to higher forms of life (Feduchi et al. 1985).

Avermectins are a series 16-membered macrocyclic lactone derivatives, obtained from the soil dwelling actinomycete *Streptomyces avermitilis*. They possess anthelmintic and insecticidal properties (Ōmura and Shiomi 2007). Eight different Avermectins were isolated as four pairs of homologous compounds. These compounds have a major and minor components ranging in ratios from 80:20 to 90:10 (Pitterna et al. 2009). Other compounds derived from the Avermectins include Ivermectin, Selamectin, Doramectin and Abamectin. The lethality of Avermectins has been attributed to their ability to block the transmittance of electrical activity in nerves and muscle cells by stimulating the release and binding of gamma-aminobutyric acid (GABA) at nerve endings. This leads to an influx of chloride ions into the cells, thereby causing hyperpolarisation and subsequent paralysis of the neuromuscular systems (Bloomquist 1993, 1996).

In agriculture, Avermectin B1 (abamectin), has been widely used for the development of formulations for the control phytophagous mites and insect pests on a variety of agricultural and horticultural crops worldwide. Abamectin is currently registered for use on ornamental plants, citrus, cotton, pears and vegetable crops as a foliar spray. It has low toxicity to non-target beneficial arthropods but it is highly unstable to light and has been shown to photodegrade rapidly on plant and soil surfaces and in water following agricultural applications. It has been found to be easily degraded by soil microorganisms, with very low levels of residues in crops. Abamectin does not persist or accumulate in the environment. Its instability combined with low water solubility and tight binding nature to soil limit Abamectin's bioavailability in non-target organisms and are considered as limiting factors for its ability to contaminate the environment (Lasota and Dybas 1990).

The Hindustan Antibiotics Limited (HAL), India has been a pioneer in the development of antibiotics for agro-usage. Two popular products manufactured by this firm are Streptocylene (a combination of Streptomycin and Tetracycline), which is a broad-spectrum systemic antibacterial antibiotic highly active against phytopathogenic bacteria and has shown effective control of various bacterial crop diseases. It is recommended for both prophylactic as well as curative applications. Aureofungin a metabolic product of *Streptovercillium cinnamomeum*, is a fungicide which is original research product of HAL. It has broad spectrum activity against various fungal infections (Dhuley et al. 1995).

Though reports of novel antibiotics from Actinobacteria are quite regular in scientific literature, reports of proper elucidation of their structure and mode of action on target and non target organisms are quite scarce. One such well characterized molecule is the metabolite 2-methylheptyl isonicotinate obtained from the culture filtrate of *Streptomyces* sp. 201. This bioactive compound, with antifungal and antibacterial activity, showed marked inhibition against dominant soil-borne phytopathogens such as *Fusarium oxysporum*, *F. moniliforme*, *F. semitectum*, *F. solani*

Table 2.2 Metabolites production by Actinobacteria in relation to plant disease suppression

Actinobacterium	Metabolite	Reference
<i>S. kasugaensis</i>	Kasugamycin	Umezawa et al. (1965)
<i>Streptomyces cacaoi</i> var. <i>asoensis</i>	Polyoxin B and D	Isono et al. (1965)
<i>S. griseochromogenes</i> 2 A-327	Blasticidin S	Kono et al. (1968)
<i>Streptoverticillium rimofaciens</i>	Mildiomycin	Iwasa et al. (1978)
<i>S. hygroscopicus</i> var. <i>geldanus</i>	Geldanamycin	Rothrock and Gottlieb (1984)
<i>S. griseus</i>	Faeriefungin	Smith et al. (1990)
<i>Micromonospora carbonacea</i>	Cellulase	El-Tarabily et al. (1996)
<i>S. olivaceoviridis</i> , <i>S. rimosus</i>	Auxins, Gibberellins,	Aldesuquy et al. (1998)
<i>S. rochei</i>	Cytokinins	
<i>S. olivaceoviridis</i> , <i>S. rimosus</i>	Amylase, Proteinase	Aldesuquy et al. (1998)
<i>S. rochei</i>		
<i>S. violaceusniger</i> YCED9	Guanidylfungin A	Trejo-Estrada et al. (1998)
<i>S. humidus</i>	Phenylacetic Acid	Hwang et al. (2001)
<i>S. padanus</i>	Fungichromin	Shih et al. (2003)
<i>S. chinaensis</i> AUBN1/7	Resistoflavin	Gorajana et al. (2005)
<i>Streptomyces</i> sp. B8005	Resistomycin and Tetracenomycin D	Kock et al. (2005)
<i>Streptomyces</i> sp. ACH505	Auxofuran (fungal growth promoter)	Riedlinger et al. (2006).
<i>Actinoplanes campanulatus</i>	β-glucanase	El-Tarabily et al. (2009)
<i>Streptomyces</i> CMU-H009	Indole Acetic Acid	Khamna et al. (2010)
<i>Streptomyces</i> sp. 5 strain	Increased Dehydrogenase activity	Stamenov et al. (2012)

and *R. solani*. The compound had no effect on seed germination and seedling development of the test plant species (Bordoloi et al. 2002). Oligomycins A and C, are macrolide antibiotics produced by the actinobacterium *Streptomyces diastaticus*, and exhibit a strong activity against *Aspergillus niger*, *Alternaria alternata*, *Botrytis cinerea* and *Phytophthora capsici* (Yang et al. 2010). The metabolites produced by Actinobacteria in relation to plant disease suppression are listed in Table 2.2.

Besides antibiotic molecules, commercial biocontrol formulations containing Actinobacteria as active ingredients are also available as tools of plant health management. Mycostop®, a biofungicide used for the control of *Fusarium* wilt of carnation and root rot disease of cucumber, contains living *Streptomyces griseoviridis* cells (White et al. 1990). Actinovate® a biocontrol formulation registered in USA, contains the actinobacterium, *S. lydicus* as its active ingredient and has been recommended for a wide range of environments ranging from green houses to turf grasses. MicroPlus® is an inoculum of *Streptomyces lydicus* WYEC 108, that has been reported to possess both disease suppression and plant growth promotion abilities. In an early report on the insect pathogenic properties of Actinobacteria, *Brevibacterium frigoritolerans* has been reported to cause bacteremia like symptoms in the soil borne larvae of the subterranean insect pests *Anomala dimidiata* and *Holotrichia longipennis*. Grub mortality occurred between the second and fifth

Table 2.3 Actinobacteria reported to possess antagonistic potential against plant pathogens

Actinobacterium	Plant pathogen	Reference
<i>S. griseochromogenes</i> 2A-327	<i>Alternaria</i> sp	Kono et al. (1968)
<i>Nocardia dassonvillei</i>	<i>Fusarium oxysporum</i> f.sp. <i>albedinis</i>	Sabaou et al. (1983)
<i>Spirillospora albida</i>	<i>Phytophthora megasperma</i> var. <i>glycinea</i>	Sutherland et al. (1984)
<i>S. hygroscopicus</i> var. <i>geldanus</i>	<i>Rhizoctonia solani</i>	Rothrock and Gottlieb (1984)
<i>Micromonospora globosa</i>	<i>Fusarium udum</i>	Upadhyay and Rai (1987)
<i>S. griseus</i>	<i>Fusarium</i> spp.	Smith et al. (1990)
<i>S. violaceusniger</i> YCED9	<i>Pythium ultimum</i>	Crawford et al. (1993)
<i>Streptomyces</i>	<i>Streptomyces scabies</i>	Liu et al. (1995)
<i>S. humidus</i>	<i>Phytophthora capsici</i>	Hwang et al. (2001)
<i>S.violaceusniger</i> strain G10	<i>Fusarium oxysporum</i> f.sp. <i>cubense</i>	Geetha and Vikineswary (2002)
<i>S. padanus</i>	<i>Rhizoctonia solani</i>	Shih et al. (2003)
<i>S.aureofaciens</i> CMUAc130	<i>Fusarium oxysporum</i>	Taechowisan et al. (2005)
<i>Actinoplanes campanulatus</i>	<i>Pythium aphanidermatum</i>	El-Tarabily et al. (2009)
<i>Kitasatospora</i> sp.	<i>F. oxysporum</i>	Moussa et al. (2011)
	<i>Phytophthora infestans</i>	
<i>Streptomyces</i> sp.	<i>Fusarium</i> sp.	Panneerselvam et al. (2012)
	<i>Alternaria</i> sp.	
<i>Streptomyces</i> sp.	<i>Xanthomonas axonopodis</i>	Poovarasan et al. (2013)

weeks after inoculation under in vitro conditions (Selvakumar et al. 2011). A non-exhaustive list of the Actinobacterial antagonists are presented in Table 2.3.

2.2.2 Actinobacteria and Plant Growth Promotion

Plant growth promotion by Actinobacteria has attracted the attention of researchers much later when compared to the well-known PGPR's. Several Actinobacteria are now reported to promote plant growth by mechanisms such as nutrient mobilization, growth hormone production, siderophore production and stimulation of beneficial rhizospheric microbes. A list of Actinobacteria with plant growth promotion traits that have been reported in the recent past are mentioned in Table 2.4.

2.2.2.1 Symbiotic Diazotrophic Actinobacteria

A unique genus amongst the Actinobacteria is *Frankia*, which has the ability to fix atmospheric nitrogen both in the free living state and in association with several tree species. When *Frankia* are associated with tree species, the association is referred to as 'Actinorhizal'. Though the actinorhizal association has been known to occur in all terrestrial ecosystems, with the exception of Antarctica (Baker and

Table 2.4 Reports of plant growth promotion by Actinobacteria

Actinobacterium	Plant growth promotion trait	Reference
<i>Streptomyces</i> sp.	Chitinase activity	Ames et al. (1989)
<i>S. thermoautotrophicus</i>	Nitrogen fixation	Gadkari et al. (1992)
<i>Streptomyces griseoviridis</i> strain K61	Growth nutrient treatment of cut flowers, potted plants and greenhouse cucumbers	Mohammadi and Lahdenpera (1992)
<i>Rhodococcus</i> sp. strain EJP75	Promotes secondary mycorrhizal lateral roots in the <i>Pinus sylvestris</i> – <i>Lactarius rufus</i> ectomycorrhizal symbiosis	Poole et al. (2001)
<i>Frankia</i>	Hydrogenases	Leul et al. (2005)
<i>S. padanus</i> AOK-30	Enhanced drought tolerance	Hasegawa et al. (2006)
<i>Arthrobacter</i> sp. strain EZB4	ACC deaminase activity	Sziderics et al. (2007)
<i>Kitasatospora</i> sp.	IAA production	Shrivastava et al. (2008)
<i>Streptomyces</i>	Solubilizes phosphate	Hamdali et al. (2008)
<i>Kitasatospora</i>	Plant growth promotion	Oliveira et al. (2009)
<i>Thermobifida</i>	Plant growth promotion	Franco-Correaa et al. (2010)
<i>Micrococcus</i> sp.	ACC deaminase activity	Siddikee et al. (2010)
<i>Rhodococcus</i> sp.	ACC deaminase activity	Francis et al. (2010)
<i>S. canus</i>	IAA, GA ₃ production	Panneerselvam et al. (2012)
<i>Streptomyces</i> strain AzR-051	IAA production	Verma et al. (2011)
<i>Streptomyces</i> sp.	Siderophore (Ferulic acid)	Schrey et al. (2012)
<i>Streptomyces</i> sp. AcM29	Siderophore (desferrioxamine B)	Schrey et al. (2012)
<i>Streptomyces</i> sp. 5 strain	Growth promotion in rye grass	Stamenov et al. (2012)
<i>Streptomyces</i> AcM 20	Increased photosynthetic yield	Schrey et al. (2012)
<i>Streptomyces</i> sp. DH6	Siderophore producer	Kaur et al. (2013)
<i>Streptomyces</i> sp. DH32	Phosphate solubilizer	Kaur et al. (2013)
<i>Streptomyces</i> sp. P2–3	Nitrogen fixer	Kaur et al. (2013)
<i>Streptomyces</i> sp. MR-14	Protease activity	Kaur et al. (2013)

Schwintzer 1990), attempts to isolate the microsymbiont, succeeded only as late as 1978 when *Frankia* was isolated in a fastidious in vitro culture (Lechevalier and Lechevalier 1990). *Frankia* strains are known to nodulate 200 actinorhizal plants, spread over 24 genera. They are divided into the following host-infection groups viz., *Alnus* and *Myrica* (Group 1), *Casuarina* and *Myrica* (Group 2), *Myrica* and *Elaeagnus* (Group 3), and those capable of nodulating members of the *Elaegnaceae* (*Elaeagnus*, *Hippophae*, *Sherpherdia*) (Group 4) (Pawlowski and Sirrenberg 2003; Roy et al. 2007). Strains belonging to the genus *Frankia* are usually not attributed a species name, due to a lack of clarity on the basis for species assignment. Hence, *Frankia alni* continues to be the only recognized species within this genus.

Morphologically *Frankia* possess hyphae, sporangia, spores and vesicles, but they do not form aerial mycelia. The vesicles are the sites of nitrogen fixation. The vesicle wall is composed of multiple lipid layers (hopanoids), and the number of layers increase with an increase in the oxygen concentration in the environment. This acts as a barrier to ambient oxygen levels, which would otherwise inhibits nitroge-nase activity. The vesicle of *Frankia* sets it apart from *Rhizobium*, which normally

fixes nitrogen in the symbiotic state, while it is protected against ambient oxygen levels, but *Frankia* can fix N_2 in the free living state as well as in association with its tree partners. Following root nodule formation and the onset of nitrogen fixation by *Frankia*, the microsymbiont derives its carbon source from plant photosynthates while the host plant benefits from the ammonium and also auxins produced by the endosymbiont (Wall and Berry 2008). The nitrogen fixed by *Frankia* in root nodules is estimated to supply 70–100% of the host plant's nitrogen requirement (Nickel et al. 2001; Myrold and Huss-Danell 2003). *Frankia* strains possess differential nitrogen fixation capabilities under in vitro and in planta conditions. The genotype of the *Frankia* strain, compatibility with the host plant, and nodule age are hypothesized to influence the level of nodule nitrogenase activity (Verghese and Misra 2000). Complex culture conditions coupled with prolonged doubling times often running into days have slowed down research into these unique organisms. But nevertheless, the contribution of the actinorhizal symbiosis, in terms of nitrogen fixation has been estimated to be as high as 25% of the annual global nitrogen fixation in terrestrial ecosystems (Dawson 2008).

The actinorhizal association begins with a compatible frankiae and host plant coming in contact. This is followed by the microsymbiont entering the host tissues by root hair deformation, penetration into the root epidermis and cortex, similar to the rhizobial—legume entry mode. But the actinorhizal nodule has a markedly distinct internal structure (Wall and Berry 2008). The lateral roots of actinorhizal plants modified by the infection process, become individual lobes. Multiple lobes make up a root nodule. The most striking feature that distinguishes the Actinobacterial nodule from the legume nodule is the proximity of infected cells to the outer periphery of the nodule lobe (Baker and Schwintzer 1990). In contrast to this, in legume nodules; layers of vascular tissue surround the infected cells, and thereby limit the exposure of nitrogenase to ambient oxygen levels. Since, Actinobacterial nitrogen fixation is confined to the highly specialized vesicle, the proximity of the infected cells to the outer periphery does not affect nitrogen fixation by Actinobacteria (Pawlowski and Sprent 2008).

2.2.3 *Actinobacteria and Recycling of Organic Matter*

The role played by Actinobacteria in the recycling of the enormous quantum of biomass generated as a result various anthropogenic activities is significant. Actinobacteria genera such as *Nocardia*, *Streptomyces* and *Micromonospora* are ubiquitous in composting processes (Waksman et al. 1939). Actinobacteria play a major role in the degradation of lignocelluloses, in the agro-environment and help return to the soil a huge quantum of plant nutrients. During the composting process a gradual increase of the temperature of the composting pile, is followed by sustained high temperatures (thermophilic) and a gradual cooling (maturation) of the composting mass (Halet et al. 2006; Hongyan et al. 2007). Microbial profiling studies during composting processes have revealed that Actinobacteria dominate during the thermophilic stage while they closely associate with fungi in the maturation stage

(Yu et al. 2007). Owing to their metabolic versatility, Actinobacteria can degrade organic molecules ranging from cellulose to complex chain lignins (Pérez et al. 2002). Very often the disease suppressive ability of composts has been attributed to the inherent Actinobacterial populations (Craft and Nelson 1996). Previous studies have suggested that the soil physical characteristics and organic matter are the main factors affecting the number and type of Actinobacteria on the soil and application of composts has been recommended to increase the Actinobacterial for both disease suppression and nourishment of the soil (Miyashita et al. 1982).

2.2.4 Actinobacteria as Plant Pathogens

Though Actinobacteria are primarily saprophytic in nature, some Actinobacteria are known to cause diseases in plants. Such Actinobacteria gain opportunistic entry into the plant system through wounds, while few others are considered as typical plant pathogens. Actinobacteria belonging to families Microbacteriaceae (*Clavibacter*, *Curtobacterium* and *Leifsonia*) and family Nocardiaceae (*Rhodococcus fascians*) are known to cause various plant pathogenic symptoms viz., galls, fasciation, gummosis, stunting and wilt in several vascular plants (Harrison 1962; Faucher et al. 1993; Boucek-Mechiche et al. 2000; Agbessi et al. 2003). The most popular of the Actinobacterial diseases is “scab” caused by members of the genus *Streptomyces*. Some Actinobacterial plant pathogens are highly host specific, a property that facilitates the quick identification of pathovars of *Clavibacter michiganensis*, *Leifsonia xyli* and *Curtobacterium flaccumfaciens* pathovars. Generally, these high-specificity pathogens do not produce symptoms on non-host plants. A typical example is the actinobacterium *Leifsonia xyli* subsp. *cynodontis* that causes stunt disease on Bermuda-grass. Though the same sub species can colonize other crop plants such as maize, rice and sugarcane, it is incapable of causing disease in non host crops (Haapalainen et al. 2000). Similarly, *C. flaccumfaciens* has been isolated as an endophyte from many crops, and some strains are known to reduce the severity of symptoms induced by the plant pathogenic *Xylella fastidiosa* when inoculated in the non-host *Catharanthus roseus* (Sturz et al. 1998; Lacava et al. 2007). Table 2.5, lists the plant pathogenic Actinobacterial species and their host range.

2.3 Actinobacterial Inoculant Technologies—Potential and Drawbacks

Though the knowledge on Actinobacteria and their metabolites have been well documented in the past, in recent times the agro-active antibiotics of Actinobacterial origin have largely remained as artefacts of academic interest and have not seen much thrust in terms of commercial exploitation, in comparison to their counterparts in the pharmaceutical sector. This can be attributed primarily due to competing interests of chemically synthesized molecules. Another factor that pre-empts the utiliza-

Table 2.5 Actinobacteria capable of causing major diseases in plants

Actinobacterium	Disease	Reference
<i>Corynebacterium poinsettiae</i> *	Bacterial canker of common poinsettia	Starr and Pirone (1942)
<i>Corynebacterium betae</i>	Silvering disease of red beet	Keyworth et al. (1956)
<i>Clavibacter michiganensis</i> subsp. <i>tessellarius</i>	Wheat leaf spot	Carlson and Vidaver (1982)
<i>Curtobacterium flaccumfaciens</i> pv. <i>oortii</i>	Yellow pock in tulip bulbs	Collins and Jones (1983)
<i>Clavibacter michiganensis</i> subsp. <i>nebraskensis</i>	Goss's bacterial wilt, blight in maize	Smidt and Vidaver (1987)
<i>Streptomyces ipomoeae</i>	Sweet potato pox	Clark and Matthews (1987); Grau et al. (2006)
<i>Streptomyces scabies</i>	Common scab of potato	King et al. (1992); Johnson et al. (2007)
<i>Clavibacter michiganensis</i> subsp. <i>insidiosus</i>	Bacterial wilt of lucerne	Paschke and Van Alfen (1993)
<i>Rhodococcus fascians</i>	Fasciations, leaf and crown gall of Various monocot and dicot plants	Eason et al. (1996)
<i>Clavibacter michiganensis</i> subsp. <i>michiganensis</i>	Bacterial canker of tomato and pepper	Dreier et al. (1997); Gartemann et al. (2008)
<i>Clavibacter michiganensis</i> subsp. <i>sepedonicus</i>	Bacterial ring rot of potato	Jahr et al. (1999); Shafikova et al. (2003)
<i>Curtobacterium flaccumfaciens</i> pv. <i>basellae</i>	Bacterial leaf spot of Malabar spinach	Chen et al. (2000)
<i>Leifsonia xyli</i> subsp. <i>xyli</i>	Ratoon stunting disease of sugarcane	Evtushenko et al. (2000)
<i>Leifsonia xyli</i> subsp. <i>cynodontis</i>	Stunt disease of Bermuda grass	Li et al. (2004)
<i>Streptomyces turgidiscabies</i>	Common scab of potato	Kers et al. (2005)
<i>Curtobacterium flaccumfaciens</i> pv. <i>flaccumfaciens</i>	Bacterial wilt of beans	Harding et al. (2007)
<i>Curtobacterium flaccumfaciens</i> pv. <i>beticola</i>	Bacterial leaf spot of sugarbeet	Chen et al. (2007)

*Originally described as *Phytomonas poinsettiae*, later classified as *Curtobacterium flaccumfaciens* (Collins and Jones 1983)

tion of Actinobacterial inoculants in the agro-environment is the perceived threat to non-target organisms and higher forms by life by the Actinobacterial secondary metabolites. Though many a times this may not be proved comprehensively, the regulatory regimes in most countries also limit the proliferation of Actinobacterial inoculants. Considering the potential of the Actinobacteria and their prevalence and dominance in the agro-environment, it would be wise to promote Actinobacterial inoculants, after comprehensive biosafety evaluation. Unlike the conventional bioinoculants that are subjected to acute exposures, a safeguard with respect to Actinobacterial inoculants, would be the evaluation of promising Actinobacteria to chronic exposure studies, in order to exclude potential biosafety issues in the future. Another feature that has excluded Actinobacteria from the bio-inoculant market, is the over emphasis on antibiotic producing genus *Streptomyces*, thereby leading to bioregulatory issues. As an alternative to this is need to explore the entire gamut

Table 2.6 Novel Actinobacteria discovered from the soil and related agro-environments in the recent past

Actinobacterium	Source	Reference
<i>Citricoccus alkalitolerans</i>	Desert soil	Li et al. (2005)
<i>Georgenia ruanii</i>	Forest soil in Yunnan (China)	Li et al. (2007)
<i>Actinomadura keratinolytica</i>	Bovine mature compost	Puhl et al. (2009)
<i>Actinopolymorpha cephalotaxi</i>	Rhizosphere soil of the plant <i>Cephalotaxus fortunei</i>	Yuan et al. (2009)
<i>Streptomyces avicenniae</i>	Rhizosphere of the mangrove plant <i>Avicennia mariana</i>	Xiao et al. (2009)
<i>Thermosporothrix hazakensis</i>	Compost	Yabe et al. (2010)
<i>Leifsonia soli</i>	Teak rhizosphere soil	Madhaiyan et al. (2010a, b)
<i>Pseudonocardia adelaidensis</i>	Surface-sterilized stem of <i>Eucalyptus microcarpa</i>	Kaewkla et al. (2010)
<i>Microbacterium azadirachtae</i>	Rhizoplane of neem seedlings	Madhaiyan et al. (2010a, b)
<i>Leucobacter exalbidus</i>	Compost	Ue (2011)
<i>Amycolatopsis dongchuanensis</i>	Soil	Nie et al. (2012)
<i>Jatrophihabitans endophyticus</i>	Surface-sterilized stem of <i>Jatropha curcas</i>	Madhaiyan et al. (2012)
<i>Actinoallomurus acanthiterrae</i>	Rhizosphere soil of the mangrove plant <i>Acanthus ilicifolius</i>	Tang et al. (2012)
<i>Amnibacterium soli</i>	Grass soil	Jin et al. (2013)
<i>Zhihengliuella flava</i>	Sea sediment	Hamada et al. (2013a, b)
<i>Spelaecoccus albus</i>	Soil of a natural cave	Lee (2013)
<i>Georgenia sediminis</i>	Sediment of the South China Sea	You et al. (2013)
<i>Streptomyces graminilatus</i>	Bamboo litter	Lee and Whang (2013)

of bacteria with high G+C content that may play a role in sustainable agriculture in near future. An indicative list of novel Actinobacteria that have been discovered over the past decade from soil and related agro-environment, are listed in Table 2.6.

2.4 Conclusion

Though Actinobacteria have colonized the agro-environment over several centuries, their role in sustainable agricultural production has remained in the backdrop, due to various reasons. But considering their multifarious abilities and the number of novel genera and species that are being added to the Phylum Actinobacteria it would not be far fetched to say that Actinobacteria are the potential inoculants of the future. But this would require the judicious screening of the Actinobacterial strains for various beneficial traits, determining their compatibility with other soil microbes and subjecting them to rigorous biosafety procedures before their eventual environmental release and commercialization. If such a system is put in place, the agro-environment would see the emergence of a new class of inoculants with far superior properties than the existing products. To achieve these there needs to be a greater coordination amongst soil microbiologists, plant pathologists and regulatory authorities the world over.

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Chapter 3

Diversity of Plant Associated Actinobacteria

Brahim Bouizgarne and A. Ait Ben Aouamar

Abstract The phylum Actinobacteria encompasses Gram-positive bacteria with a high DNA G+C. In soils, they present multiple lifestyles: rhizosphere saprophytes, endophytes, facultative symbionts and obligate phytopathogens. They play either beneficial or adverse effects towards plants. Numerous studies focused on their taxonomy and preservation. Also, adequate strategies were developed to enable their isolation. Phenotypically, the Actinobacteria is one of the most diverse phyla within bacteria. Their presence was once monitored by classical culture dependent techniques and phenetic characterization. Development of culture independent methods including chemotaxonomic and genomic approaches such as 16S rRNA gene analysis allowed to detect the so called unculturable bacteria. Major works on their diversity combine culture dependant and independant techniques. This chapter focuses on the phenetic and genetic diversity of Actinobacteria in plant-soil systems. It begins with some knowledge of their biology and their taxonomy, followed by a brief overview of the methods that have facilitated advances in the understanding of their diversity. It also discusses diversity of ecologically important plant associated Actinobacteria (rhizosphere and phyllosphere colonizers, endophytes, symbionts and phytopathogens).

3.1 Introduction

The phylum Actinobacteria belongs to reign of prokaryota, domain of Bacteria, division of Firmicutes (Gram-positive filamentous bacteria). Stackebrandt et al. (1997) on the basis of molecular and chemical taxonomy, proposed the class Actinobacteria. According to the Bergey's Manual of Systematic Bacteriology (Volume 5), the phylum is divided into six classes: Acidimicrobiia, Rubrobacteria, Coriobacteriia, Nitrospirae, Thermoleophilia and Actinobacteria (Ludwig et al. 2012). The class Actinobacteria encompasses Gram-positive filamentous bacteria with

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high Shagraff coefficient (C+G or DNA cytosine and guanine percentage) which is above 70%. Actinobacteria are aerobes but certain species are facultative. Actinobacteria (also known as actinomycetes) comprises 16 orders and 43 families. Orders are: Actinomycetales (one family, Actinomycetaceae), Actinopolysporales (one family, Actinopolysporaceae), Bifidobacteriales (one family, Bifidobacteriaceae), Catenulisporales (two families, Catenulisporaceae and Actinospicaceae), Corynebacteriales (six families, Corynebacteriaceae, Dietziaceae, Mycobacteriaceae, Nocardiaceae, Segniliparaceae, and Tsukamurellaceae), Frankiales (six families, Frankiaceae, Acidothermaceae, Cryptosporangiaceae, Geodermatophilaceae, Nakamurellaceae, and Sporichthyaceae), Glycomycetales (one family, Glycomycetaceae), Jiangellales (one family, Jiangellaceae), Kineosporiales (one family, Kineosporiaceae), Micrococcales (fifteen families, Micrococcaceae, Beutenbergiaceae, Bogoriellaceae, Brevibacteriaceae, Cellulomonadaceae, Dermabacteraceae, Dermacoccaceae, Dermatophilaceae, Intrasporangiaceae, Jonesiaceae, Microbacteriaceae, Promicromonosporaceae, Rarobacteraceae, Ruaniaceae and Sanguibacteraceae), Micromonosporales (one family, Micromonosporaceae), Propionibacteriales (two families, Propionibacteriaceae and Nocardiodaceae), Pseudonocardiales (one family, Pseudonocardiaceae), Streptomycetales (one family, Streptomycetaceae), Streptosporangiales (three families, Streptosporangiaceae, Nocardiospiceae, and Thermomonosporaceae) and the order Incertae sedis (genus *Thermobispora*). The class Actinobacteria represents one of the largest taxonomic units among the major lineages within the domain Bacteria. The phylogenetic relationships of taxa above the genus level (e.g., families) are based only on specific 16S rRNA signatures. However, classification at generic and species levels also takes into account the phenotypic features. The name “Actinomycetes” was derived from Greek “*atki*” (a ray) and “*mykes*” (fungus) due to their ability to form mycelia which resemble those characteristics of fungi. Indeed, one of the major traits of the class Actinobacteria is the presence of filamentous mycelia structures with frequent presence of aerial mycelium bearing conidia. However, they are real prokaryotes without nuclear membrane. Filaments diameter is similar to that of bacteria. Since the discovery of the antibiotic actinomycine from a culture of *Streptomyces antibioticus* (Waksman and Woodruff 1940) and of streptomycine from *Streptomyces griseus* (Shartz et al. 1944), Actinobacteria laid the platform to search for novel biotechnologically significant bioactive secondary metabolites and especially antibiotics and are now-a-days of great biotechnological interest albeit the great progress in chemical synthesis. Indeed, 75% of known antibiotics are naturally produced from Actinobacteria and particularly those belonging to the genus *Streptomyces*. They also hold a prominent position due to their ability to produce diverse other secondary metabolites of commercial interest (antivirals, antitumor agents, immunosuppressors, biopesticides, anti-helminthic agents, enzymes etc.).

Actinobacteria constitute a very diverse class, forming a group of bacteria well adapted to various ecosystems. They colonize geographically distinct aquatic (river waters, estuaries), marine ecosystems (Giovannoni and Stingl 2005) and even the arctic areas and are able to colonize all types of terrestrial ecosystems (mountains, agricultural and forest soils). They are even found in marine deep sediments (Mal-

donado et al. 2005) and extreme environments (Hirsch et al. 2004). Trophically, they belong to all known trophic types on earth: energy chemotrophs, phototrophs, carbon source autotrophic and heterotrophic but majority are saprophyte chemoheterotrophs able to use a great variety of carbon molecules for their energy including most recalcitrant complex polymers (Lechevalier 1988; Zimmerman 1990). Generally, it is difficult to dissociate Actinobacterial diversity from that of the whole bacterial diversity in an ecosystem. Actinobacteria are widely distributed in terrestrial ecosystems, where they contribute to soil nutrient cycling and live in association with plants. Extensive researches on their diversity in soils and particularly in agro-systems have been performed. Cultivation and molecular analyses of Actinobacteria sampled from a range of soil depths and around numerous plant root systems has expanded the diversity of Actinobacteria which has been detected and extended our knowledge on their classification, their functioning and interactions with other microorganisms and plant species.

Over the last decades, a huge number of works aiming to screen for new species and new metabolites of interest have been developed. Now-a-days, with the development of modern techniques, Actinobacterial systematics gradually evolved to polyphasic taxonomy taken into account chemotaxonomy and molecular taxonomy. Like all other bacteria, nomenclature of Actinobacteria is governed by the International Code of Nomenclature of Bacteria and Approved List of Bacterial Names (Skerman et al. 1980; Lapage et al. 1992; Euzéby and Tindall 2001). Their characterization based on the nature of mycelia and spores is the simplest approach to their study and classification. Simple morphology is represented by species without mycelia such as coccoid forms (*Micrococcus*), rod-coccoid (e.g., *Arthrobacter*), coryneform (e.g., *Clavibacter* and *Leifsonia*) to the more complex filamentous forms differentiated as aerial and substrate mycelium represented by species with permanent hyphae (e.g., *Streptomyces*) or fragmenting hyphal structures (e.g., *Nocardia*). For example, representatives of the genus *Streptomyces* have filaments of 0.5–2.0 μm (Loria et al. 2003). The majority of Actinobacteria develop vegetative mycelium also called substrate mycelium which is surmounted by aerial mycelium. The latter is frequently of powdery or downy aspect. The two types of mycelia could present various colors: white, gray, yellow, red, blue, orange etc. On the other hand, spores (conidia) represent a fundamental criterion for the taxonomy of Actinobacteria (Locci and Sharples 1984). They represent the major elements of conservation in hostile environment conditions (Lechevalier 1988; Zimmerman 1990) and are not formed in coryneforms such as *Clavibacter michiganensis* and *Leifsonia*. For filamentous forms, their disposition, either directly on sporophores or into vesicles and sporangia is considered for their recognition. Some Actinobacteria have aerial mycelium bearing short spore chains, such as *Actinomadura* while others such as *Streptomyces*, *Nocardoides* and some *Nocardia* present long spore chains. Spore chains of the Genus *Streptomyces* surmount monopodial aerial hyphae and some species (formerly called *Streptoverticillium*) have verticillate sporophores. *Streptomyces* spore chains may be straight (rectis type), flexous (rectiflexibilis type), looped (retinaculiperti type) or helicoïdal (spirales type) and may exhibit five possible shapes (smooth, warty, spiny, hairy and rugose) (Dietz and

Mathews 1971). Sporangia containing non motile spores are found in the genus *Frankia* and *Streptosporangium* and Sporangia with motile spores (zoospores) in the genera *Actinoplanes*, *Ampulariella*, *Pilimelia*, *Planomonospora*, *Planobispora*, *Dermatophylus*, *Geodermatophylus* and *Dactylosporangium*. Vesicle containing spores are characteristic of the non-leguminous Nitrogen fixing actinobacterium *Frankia*. For groups lacking aerial mycelium, spores are formed directly on the substrate mycelium (e.g., *Micromonospora*, *Actinoplanes*, and *Ampulariella*). Some Actinobacteria form single small spores (*Micromonospora*, *Saccharomonospora* and *Promicromonospora* and *Thermomonospora*) or more than one spore (*Microbispora* and *Microtetraspora*).

For studies, aiming to identify Actinobacteria and to assess their diversity, phenotypic traits are of great interest. However, usually, more than one method is used i.e., phenotypic and genotypic method. These methods are either Culture-based techniques or Culture-independent techniques. For extensive review on advantages and drawbacks of these methods, please refer to Kirk et al. (2004) and Rastogi and Sani (2011).

3.2 Methods to Study Diversity of Actinobacteria

Actinobacteria, with great diversity, recently studied by the two broad means; culture-based techniques and culture-independent techniques and are discussed below:

3.2.1 Culture-Based Techniques to Study Actinobacterial Diversity

Culture-based techniques consider culturable diversity of Actinobacteria that involves standard microbiological method and characterization. These studies have immense importance in exploration of Actinobacterial diversity.

3.2.1.1 Plate Count and Morphological Approaches

Plate count technique represents the simplest method to assess the diversity of Actinobacteria. It is performed by using selective plating and direct colony counts. Cultivation of Actinobacteria sampled from a range of soil depths or in the vicinity of plant roots is performed by plating on selective media. For rhizospheric soil Actinobacteria, soil is pretreated with air drying, desiccation, heat treatments and sonication. While it is relatively easy to isolate Actinobacteria from soil samples, endophytes and symbionts require root or stem surface sterilization as pretreatment (Lechevalier and Lechevalier 1990; Tian et al. 2007; El-Shatoury et al. 2013). For plating soil Actinobacteria, usually the selective media are supplemented with an-

tibiotics such as cycloheximide and nalidixic acid which prevent the proliferation of most fungi and Gram-negative bacteria. Isolating the symbiotic *Frankia* from non leguminous plant nodules is relatively fastidious. They are isolated either from soil samples (Maunuksela et al. 1999) or from crushed roots after surface sterilization (Baker and O'Keefe 1984; Rosbrook et al. 1989; Baker 1990; Lechevalier and Lechevalier 1990). Their growth needs special procedures (Baker and O'Keefe 1984; Sayed and Wheeler 1999; Bassi and Benson 2007). Most abundant Actinobacteria in soils are *Streptomyces* and *Nocardia*. Rare Actinobacteria require enrichment or special physical and chemical pretreatments and use of selective antibiotics. They include *Actinoplanetes*, *Actinomadura*, *Amycolatopsis*, *Dactylosporangium*, *Kineosporia*, *Microbispora*, *Microbispora*, *Micromonospora*, *Microtetraspora*, *Nocardia*, *Saccharomonospora*, *Thermomonospora*, *Pseudonocardia* and *Streptosporangium*. Hayakawa (2008) reviewed methods for their isolation, enumeration and distribution in soil. Actinobacteria are recognized on Petri Plates according to their colony characters and microscopy observations for mycelia and spores. The color of aerial and substrate mycelium, production of diffusible and melanoid pigments are criteria for the classical taxonomy of Actinobacteria. Light microscopy, TEM or SEM (Williams and Fisher 1970) could be used for observation of the filaments and spores.

3.2.1.2 Biochemical and Physiological Approaches

In addition to morphology, biochemical-based techniques are also used to identify and study their diversity. Biochemical tests include growth conditions such as pH tolerance and temperature and resistance to growth inhibitors. Community level physiological profiling (CLPP), also known as sole-carbon-source utilization (SCSU) (Garland and Mills 1991; Bouček-Mechiche et al. 1998; Grayston 2000; Ibekwe et al. 2001; Grayston et al. 2004; Kaneshiro et al. 2006; Flores-González et al. 2008) is based on the ability of Actinobacteria to grow on different carbohydrates. Some miniaturized systems used initially for other phyla proved to be useful for the identification of Actinobacteria. A system such as commercial Biolog GP2 MicroPlate is designed for identification and characterization of a very wide range of aerobic Gram-positive bacteria and was used for Actinobacteria (Ibekwe et al. 2001). It contains 95 different carbon sources and one control well without a substrate. Metabolism of the carbohydrate is detected by color change following reduction of tetrazolium, Biolog GP gives a characteristic reaction pattern called a "metabolic fingerprint." Physiological fingerprints with the api® stripes (bioMérieux) are determined by using for example the API Coryne could be used for the identification of some Actinobacteria (Trujillo et al. 2006b). The API Zym (Trujillo et al. 2006a) and API 20E, are also rapid systems for the identification of Actinobacteria based on bacterial enzyme activities (Smith et al. 1972; Humble et al. 1977). CLPP method is useful when studying functional diversity of soil and plant associated Actinobacteria. It represents a valuable tool usually used in combination with molecular techniques. However, more than 99% of the microorganisms observed

through a microscope are not cultivable. Thus, culture techniques had the disadvantage of giving limited and biased data on microbial diversity. Consequently, informations on unculturable Actinobacteria constitute a loss of informations about the overall real activities in the soil environment.

3.2.2 *Culture-Independent Techniques to Study Actinobacterial Diversity*

Culture-independent techniques consider culturable diversity of Actinobacteria and involve chemical and genotypic approach.

3.2.2.1 Chemical Approaches

Chemical traits are also used for the identification of Actinobacteria. According to Goodfellow and Minnikin (1985) chemotaxonomy uses chemical features for the classification as alterations in morphological features are the results of chemical alterations (O'Donnell 1988). For the Actinobacteria, chemotaxonomy is based on bacterial cell wall amino-acids (Lechevalier and Lechevalier 1980), membrane fatty acids (Kroppenstedt 1985), phospholipids (Lechevalier and Lechevalier 1980), mycolic acids (Hecht and Causey 1976), menaquinones (Kroppenstedt 1985) and cell sugars (Lechevalier and Lechevalier 1980). Chemical approaches could be used to identify Actinobacteria after cultivation but also as culture independent approach for studying their diversity (Clegg et al. 2003). The peptidoglycan type or the nature of cell wall amino-acids allowed distinguishing between groups of Actinobacteria. Isomers of the diaminopimelic acid (DAP) are considered for the determination of major chemotypes. Diaminopimelic acid (DAP) is a major constituent of Actinobacteria cell walls and is represented by two isomers LL and DL (meso). According to DAP form, amino acids and sugars, eight chemotypes are adopted (chemotype I to VIII wall sensu) (Lechevalier and Lechevalier 1970). Chemotype I: LL-DAP (LL-A₂pm, glycine and no sugars) is found in the species *Streptomyces* (*Streptomycetacea*), *Nocardoides* (*Nocardiodaceae*), *Intrasporangium Terabacter* (*Intrasporangiaceae*) and *Sporichthya* (*Sporichthyaceae*); chemotype II (meso (LD) DAP (meso-A₂pm), glycine, arabinose and xylose) is represented by *Micromonospora*, *Actinoplanes*, *Catellatosporia*, *Dactylosporangium*, *Ampulariella* and *Pilimelia* (family *Micromonosporaceae*) and *Glycomyces* (*Glycomycetacea*); chemotype III: meso DAP and madurose (3-O-methyl-D-galactose) is represented by *Actinomadura Actinocorallia* and *Thermomonospora* (family *Thermomonosporaceae*); *Streptosporangium*, *Microbispora*, *Microtetraspora*, *Nonomuraea*, *Planobispora*, *Planomonospora* and *Planotetraspora* (family *Streptosporangiaceae*); *Dermatophylus* (*Dermatophylaceae*) and some *Frankia* (*Frankiaceae*). Most *Frankia* possess xylose or fucose as cell sugar constituents, chemotype IV: meso DAP, arabinose and galactose, is represented by *Nocardia* and *Rhodococcus* (family *Nocardiaceae*) and *Pseudonocardia*, *Actinobispora*, *Amycolatopsis*, *Saccharomonospora* and *Saccharopolyspora* (family *Pseudonocardiaceae*). The other chemotypes (V to VIII)

are deprived of DAP and possess other diamino acids as cell wall major amino acid constituent (e.g., *Micrococcus*, *Arthrobacter*, *Kocuria* and *Rothia*, family *Micrococcaceae*). DAP analysis is one of the major traits of the taxonomy of Actinobacteria. However, diverse Actinobacteria share the same DAP profile. Thus, for assessing the phenotypic diversity of Actinobacteria, it should be used in combination with other phenotypic or genotypic criteria.

Fatty acid methyl esters (FAMES) (Simon et al. 1989; Paradis et al. 1994), menaquinones (Kroppenstedt 1985) and phospholipid fatty acid (PLFA) (Lechevalier and Lechevalier 1980) analyses are important tools for the identification of Actinobacteria. Lechevalier and Lechevalier (1980) distinguish five phospholipids groups and four groups based on FAME analysis. For example, the majority of *Streptomyces* have straight chain, iso- and anteiso-branched chain fatty acids with a carbon chain-length of 14–18 atoms (Saddler et al. 1987). FAME analysis could be used as a culture-independent method to assess bacterial community structures (Cavigelli et al. 1995; Ibekwe and Kennedy 1998). Soil samples could serve to detect the presence of Actinobacteria. In this case, FAME profiles, could unfortunately derive from bacteria but also from humic materials, as well as plant and root exudates. Also, dead cells could lead to over estimations of the communities. Phospholipid fatty acids have the advantage of being exclusively constituents of microbial cell membranes. They could be directly extracted from soil samples. Actinobacteria show different phospholipid patterns (PI to PIV sunsu) (Lechevalier et al. 1981): PI type (phosphatidylglycerol with phosphatidylinositol, phosphatidylinositol mannosides) is found in *Actinomadura*, *Glycomyces*, *Frankia* and *Nocardoides*. The genera *Streptomyces*, *Kitasatospora*, *Actinoplanes*, *Ampullariella*, *Geodermatophilus*, *Micromonospora*, *Dactylosporangium* and *Pilimelia*, possess PII phospholipid type (phosphatidylethanolamine). PIII; phosphatidylcholine (with phosphatidylethanolamine, phosphatidylmethylethanolamine and phosphatidylglycerol) is found in *Kineospora*, *Nocardiopsis* and *Pseudonocardia* and PIV; glucosamine (with phosphatidylethanolamine and phosphatidylmethylethanolamine) in *Microbispora*, *Microtetraspora*, *Planomonospora* and *Planobispora*. In assays aiming monitoring microbial diversity, the PLFA profiles of 10Me18:0 is indicative of the Actinobacteria. In general, PLFA technique is valuable for assessing microbial diversity as unique fatty acids are indicative of specific groups of bacteria. PLFA method allowed assessing to a greater proportion of the microbial community resident in soil than culture methods and has been used as a culture-independent method of assessing the structure of microbial communities (Ibekwe and Kennedy 1998; Clegg et al. 2003; Grayston et al. 2004; Clegg 2006). In addition, this method allow to detect differences in the composition and behavior of Actinobacterial communities in plant systems under various soil and crop practices (Fritze et al. 2000; Ibekwe et al. 2001; Christopher et al. 2003; Grayston et al. 2004; Clegg 2006; Qi-chun et al. 2007).

3.2.2.2 Genotypic Approaches

The phenotypic approach cited above, though was the first to be used for the classification of Actinobacteria, it still not sufficient to establish a rational clustering of Actinobacterial genera below genus level classification. It fails to distinguish between

closely related organisms (Goodfellow et al. 1988). Stackebrandt et al. (1997) have noted that phenotypic diversity of phylogenetically closely related genera makes the description of families and higher taxa so broad and useless for the description of the enclosed taxa and consequently, phylogenic differentiation by non-genotypical methods remains limited. As the mechanisms responsible of divergence of bacterial species are changes in genomic composition, culture independent molecular techniques have been devised to overcome the stumbling blocks associated with the presence in soil of unculturable microorganism which are not detected by the culture method (Ward et al. 1995; Rheims et al. 1996; Felske et al. 1997). Genotypic methods allowed reconsideration of the taxonomy of Actinobacteria. The taxonomical position of several genera and species were then re-evaluated. Currently, most of taxonomist agree that the polyphasic approach combining phenotype, chemotaxonomy and genotypy should be considered for accurate identification of isolates (Vandamme et al. 1996). Using molecular techniques, extensive diversity in plant associated Actinobacteria was detected.

16S rDNA Sequencing Most used techniques are based on the ribosomal 16S rDNA and rRNA (Rintala et al. 2001; Sessitsch et al. 2002; Clawson et al. 2004; Carro et al. 2013) which had the advantages to hold both conserved and highly variable regions (Woese 1987) that permits the discrimination of bacteria and allow a fine taxonomy below the genus level. This was rendered easier with the availability of sequences in renowned public-domain databases that allow estimating phylogenetic relatedness between ready to identify bacterium and all available type strains in databases. To assess Actinobacterial diversity, DNA could be isolated from soils (van Elsas and Smalla 1995; Felske et al. 1996; Clegg et al. 2003; Clegg 2006) plant tissues (Benson et al. 1996; Tian et al. 2007; Nimnoi et al. 2010) or from pure Actinobacterial cultures after cultivation (Wanner 2007a, b; St-Onge et al. 2008; El-Shatoury et al. 2013) and serve as the template for amplification of 16S rDNA by PCR technique. Universal primers (Weisburg et al. 1991) could be used. The design of taxon-specific oligonucleotide probes is valuable as selective amplification primers could led to an effective and rapid identification of large numbers of actinomycetes. Thus, specific oligonucleotide primers targeting Actinobacteria were developed (Heuer et al. 1997; Rintala et al. 2001; Monciardini et al. 2002; Stach et al. 2003) and used for selective isolation and characterization of targeted genera (Salazar et al. 2000; Rintala et al. 2001; Monciardini et al. 2002; Salazar et al. 2002; Tan et al. 2006) or even species (Dreier et al. 1995; Morón et al. 1999; Song et al. 2004b). PCR products could subsequently serve to obtain partial or total rRNA (or rDNA) sequences for phylogenetic analyses (Niner et al. 1996; Benson et al. 1996; Song et al. 2004b; Wanner 2007a, b, 2009; Janso and Carter 2010). Currently, sequencing of almost complete 16S rDNA sequence (about 1500 bp) is the most accepted gene size for accurate identification of Actinobacteria. Sequences are then compared to those provided by easily accessible databases (e.g., Genebank) for phylogenetic studies. Comparison could be performed for example by the BLAST tool (Altschul et al. 1990; Zhang et al. 2000). Although labor-intensive and time-consuming, clone libraries method based on rDNA or 16S rRNA genes

(rDNA) where cloning and sequencing of rDNAs from soil samples or plant tissues is largely used (Macrae et al. 2000; Kaiser et al. 2001; Sessitsch et al. 2002).

Genomic Fingerprinting Methods These are restriction fragment length polymorphism (RFLP), Terminal restriction fragment length polymorphism (T-RFLP) and ITS-RFLP. PCR products could serve for RFLP profiling also known as ARDRA. In this technique, fragments of amplified or cloned ribosomal DNA genes, cut with restriction enzymes are analyzed on the basis of the polymorphism in the length of RFLP (Clegg et al. 2003; Clegg 2006; Nimnoi et al. 2010). T-RFLP is a variant of RFLP where one PCR primer is labeled with a fluorescent dye, allowing detection of only the labeled terminal restriction fragment. Study of polymorphism of fluorescently labeled terminal restriction fragment of 16S gene within Actinobacteria populations is made easier than with RFLP. This technique was used to identify Actinobacteria and to analyze their populations (Sessitsch et al. 2002; Conn and Franco 2004). Intergenic variable spacers regions (IGS) are highly variable non-coding part of DNA. Internal transcribed spacer region (ITS) between the 16S and 23S rRNA, are excised and degraded during maturation process of transcription and, thus, show more variation than adjacent rRNA genes. They provide a high potential in discriminating closely related species because of high sequence and length variations in this region. ITS-RFLP consists of analyzing sequences of the 16S–23S rRNA internally transcribed spacer (ITS). Lanoot et al. (2005), found good correlation between 16S-ITS RFLP fingerprinting and 16S rDNA sequencing phylogeny in Actinobacteria. Moreover, these authors showed that taxonomic resolution of ITS RFLP is higher than that of 16S rDNA sequencing to delineate phylogenetically related species. Hence, these authors concluded that ITS RFLP could be used as an alternative to the laborious DNA–DNA hybridization. This technique was used to assess diversity within populations of Actinobacteria (Song et al. 2004b; Wanner 2009; de Leon et al. 2009; Dees et al. 2012). Many researches focused on the IGS between functional genes in Actinobacteria such as *nif* gene (Jamann et al. 1993; Navarro et al. 1997; Hahn et al. 1999; Dai et al. 2004). Random amplified polymorphic DNA (RAPD) uses short primers, which anneal randomly at multiple sites on the genomic DNA under low annealing temperature and generates PCR amplicons of various lengths, was also used for some Actinobacteria (Patrik and Rainey 1999; de Leon et al. 2009). Amplified Fragment Length Polymorphism (AFLP) is a recent technique which combines the advantages of RAPD and RFLP was applied to of some Actinobacteria for studying biodiversity (de Leon et al. 2009). PCR products could also serve for fingerprinting techniques such as Denaturing gradient gel electrophoresis (DGGE) and Temperature gradient gel electrophoresis (TGGE). Community analysis of Actinobacteria by using PCR amplification of the 16S rRNA gene (rDNA) in combination with DGGE or TGGE is commonly performed in microbial ecology (Heuer et al. 1997; Miller et al. 1999; Smalla et al. 2001; Sessitsch et al. 2002; Nimnoi et al. 2010). DNA could be extracted from soil, plant tissues or from bacterial cells (Miller et al. 1999; Smalla et al. 2001; Nimnoi et al. 2010) before DGGE. DNA is subsequently PCR-amplified for 16S or 18S rRNA sequences to study polymorphism in a linear denaturing gradient (increasing concentration of denaturants) for DGGE or temperature denaturing gradients for

TGGE. PCR-DGGE relies on available actinomycete-specific primers and is very reliable and sensitive as it can separate DNA with one base-pair difference. Group-specific PCR-DGGE systems are now available for studying actinomycetes (Heuer et al. 1997). As to get a phylogenetic insight into the diversity of Actinobacteria in rhizosphere and bulk soil, DGGE allows to a cultivation-independent analysis of large numbers of Actinobacteria in a sample.

Highly Repeated Sequence PCR Fingerprinting (rep-PCR) Many Actinobacteria possess highly repetitive DNA sequences repeated throughout their genomes. These sequences may allow differentiation down to the species or strain level. rep-PCR, provides genomic fingerprint of chromosome structure (Rademaker and De Bruijn 1997). Thus, it has been used for differentiation at species and subspecies level (Sadowsky et al. 1996; Clark et al. 1998; Louws et al. 1998; Jeong and Myrold 1999; Smith et al. 2001; Lanoot et al. 2004; Nazari et al. 2007; de Leon et al. 2009; Trujillo et al. 2010). This technique is simple, rapid and inexpensive and allows grouping closely related organisms according to their fingerprints and separating them from distant groups.

Nucleic Acid Hybridization Techniques DNA–DNA hybridizations are based on experiments of reassociation of single-stranded DNAs from different organisms that form a heterologous hybrid (Labeda 1992; Kieser et al. 2000). This method has been used to determine species identity of Actinobacteria. In bacterial taxonomy, it is commonly accepted that if two strains share 70% DNA–DNA homology or greater, they are considered to be related at the species level. The notion of species was then defined as a group of strains, including the type strain, that share such values of DNA–DNA relatedness with a difference in the melting temperature (ΔT_m) of 5°C or less between the homologous and heterologous hybrids formed (Wayne et al. 1987). Total DNA–DNA hybridization proved to be a powerful tool to study diversity among groups of Actinobacteria (Zaluga et al. 2013). However, this technique has also the disadvantage of being fastidious and time consuming. In situ hybridization includes Direct whole cell hybridization (Hahn et al. 1997; Zepp et al. 1997a) and fluorescent in situ hybridization (FISH). Actinobacterial commune study by FISH and scanning confocal laser microscopy (SCLM) has also been used for direct identification and quantification of Actinobacteria within their natural microhabitat. This method use microscopic observation of in situ 16S or 23S rRNA hybridized with fluorescently-labeled-specific oligonucleotide (MacNaughton et al. 1994). This method has the advantage of directly targeting whole cell. So biases arising from DNA extraction, PCR amplification, and cloning are avoided (Ludwig et al. 1997).

Housekeeping Genes Another valuable method is the genetic analyses of the housekeeping genes such as *rpoB* (the RNA polymerase β subunit-encoding gene), *rpoD*, *gyrB* (gyrase subunit β gene), *recA* (encoding a protein involved in repairing damaged DNA in the SOS regulon) and multilocus sequence analysis MLSA (Dahllöf et al. 2000; Kasai et al. 2000; Maréchal et al. 2000; St-Onge et al. 2008; Bernèche-D'Amours et al. 2011; Waleron et al. 2011; Jacques et al. 2012; Carro et al. 2012; Xu et al. 2012b).

3.3 Diversity of Actinobacteria Associated to Plant Soil Systems

In soil, Actinobacteria represents a high proportion of the microbial biomass. Their populations are found at 10^6 – 10^9 bacteria g^{-1} (Kuster 1968; Goodfellow et al. 1968) and they represent more than 30 % of total population of soil microorganisms (Kennedy 1999). However, analyses of Actinobacteria isolated from several plant associated niches reveal a larger diversity than that obtained by culture dependant methods (McVeigh et al. 1996; Takeuchi et al. 1996; Conn and Franco 2004). Actinobacteria are extremely abundant in soils where they grow as hyphae and share the biotope with other telluric micro-organisms like fungi. Their vertical repartition in soil ranges from surface to up to 2 m depth (Breton et al. 1989). Their populations are abundant in surface layer and gradually decrease with the depth. In soil, they are the origin of the characteristically “earthy” smell of freshly turned healthy soil due to the production of the terpenoid geosmin (Gerber and Lechevalier 1965; Scholler et al. 2002). Two major Actinobacterial genera are well represented in soil, *Streptomyces* and *Nocardia*. *Streptomyces* could alone represent about 95 % of the whole soil Actinobacterial micropopulation (Williams and Vickers 1988). Actinobacteria play a major role in agricultural soil quality promotion by contributing to soil fertility (Goodfellow 1984). By their filamentous structure and their production of extracellular polysaccharides, Actinobacteria bind soil particles together and thus contribute to maintaining soil structure (Kennedy 1999). Actinobacteria decompose complex mixtures of soil polymers (Lechevalier 1988; Zimmerman 1990). They could degrade organic substances including proteins, polysaccharides, starch, keratins, celluloses, lignocelluloses and lignins. They were found to be involved in carbon and nitrogen cycle (McCarty and Williams 1992). Thus, their activities are conducive to crop production. During the last decades, an increasing interest has emerged with respect to the importance of Actinobacterial diversity in soil habitats, particularly Actinobacteria as a part of soil microbial community that strongly influence on plant biology. Representative species of the main subdivisions of the class Actinobacteria, found to be associated with plants in soil shown in Table 3.1. They include Actinobacteria isolated from rhizospheric soils and rhizoplane and from internal plant tissues (endophytes, symbionts of actinorhizal plants and plant pathogens).

3.3.1 Rhizosphere Associated Actinobacteria

The rhizosphere defined as the soil volume surrounding the plant roots and influenced by the plant root (Sørensen 1997). Due to the availability of substrates for metabolism, the rhizosphere could support large populations of microorganisms such as bacteria, actinomycetes, fungi, protozoa and algae etc. Actinobacteria are aggressive root and rhizosphere colonizers. Their diversity in the vicinity of root plants have been assessed by culture dependant methods such as plate counts

Table 3.1 Taxonomic subdivisions of the class Actinobacteria and representatives of validly published species associated with plants^a

Order	Family	Main genera	Species	Plant	Reference
Catenulisporales	Catenulisporaceae	Catenulispora	<i>C. graminis</i>	Rhizosphere soil of <i>Phyllostachys nigro</i> <i>var. henonis</i> (bamboo)	Lee et al. (2012)
			<i>N. artemisiae</i>	Endophytic, <i>Artemisia annua</i>	Zhao et al. (2011c)
			<i>N. callitridis</i>	Endophytic, <i>Callitris preissii</i> (Austrian native pine tree)	Kaewkla and Franco (2010a)
Corynebacteriales	Nocardiaceae	<i>Nocardia</i>	<i>N. vaccinii</i>	Pathogen, <i>Blueberry</i> gall	Demaree and Smith (1952)
			<i>R. artemisiae</i>	Endophytic, <i>Artemisia annua</i>	Zhao et al. (2011d)
			<i>R. fascians</i>	Pathogen, <i>Leafy gall</i> and <i>Fasciation</i> and stunting of various plants including tobacco	Vereecke et al. (2003) Goethals et al. (2001)
Frankiales		<i>Gordonia</i>	<i>R. kunmingensis</i>	Rhizosphere of <i>Taxus chinensis</i>	Wang et al. (2008b)
			<i>G. rhizosphaera</i>	Rhizosphere of <i>Bruguiera gymnorrhiza</i> (mangrove)	Takeuchi and Hatano (1998)
		<i>Williamsia</i>	<i>W. muralis</i>	Endophytic, <i>Eucalyptus microcarpa</i> (Grey Box) and <i>Pittosporum phylliraeoides</i> (native apricot tree)	Kaewkla and Franco (2013c)
			<i>W. sterculiae</i>	Endophytic, stems of chinese medicinal plants	Fang et al. (2013)
		<i>Dietzia</i>	<i>D. schimae</i>	Endophytic, <i>Schima</i> sp.	Li et al. (2008)
			<i>D. cercidiphylli</i>	Endophytic, <i>Cercidiphyllum japonicum</i>	Li et al. (2008)
		<i>Frankia</i>	<i>F. alni</i>	Symbiotic, N2 fixing symbionte of actinorhizal plants	Normand et al. (1996)
			<i>G. ruber</i>	Rhizosphere soil of <i>Astragalus membranaceus</i>	Zhang et al. (2011)
		<i>Blastococcus</i>	<i>B. endophyticus</i>	Endophytic, leaves of <i>Campiotheca acuminata</i>	Zhu et al. (2013)
			<i>G. endophyticus</i>	Endophytic, roots of <i>Carex baccans</i>	Qin et al. (2008b)
Glycomycetetales	Glycomycetaceae	<i>Glycomyces</i>	<i>G. mayeni</i>	Endophytic two medicinal plants	Qin et al. (2009a)
			<i>G. sambucus</i>	Endophytic, stem of <i>Sambucus adnata</i>	Gu et al. (2007)

Table 3.1 (continued)

Order	Family	Main genera	Species	Plant	Reference
Jiangellales	Jiangellaceae	Jiangella	<i>G. scopariae</i>	Endophytic two medicinal plants	Qin et al. (2009a)
			<i>J. alba</i>	Endophytic, <i>Maytenus austroyunnanensis</i>	Qin et al. (2009e)
Kineosporiales	Kineosporiaceae	Kineosporia	<i>K. succinea</i>	Endophytic, Leaves of <i>Typha latifolia</i>	Kudo et al. (1998)
			<i>K. rhamnosa</i>	Endophytic, Leaves of <i>Typha latifolia</i>	Kudo et al. (1998)
			<i>K. rhizophila</i>	Endophytic, Root of <i>Cyperus nziroiria</i> (galingale)	Kudo et al. (1998)
			<i>K. rhizosphaerae</i>	Rhizosphere of <i>Peucedanum japonicum</i>	Lee (2009b)
Micrococcales	Micrococcaceae	Kineococcus	<i>K. gynurae</i>	Endophytic, Roots of <i>Gynura pseudo-china</i> var. <i>hispida</i>	Duangmal et al. (2008)
			<i>M. endophyticus</i>	Endophytic, <i>Aquilaria sinensis</i>	Chen et al. (2009b)
		Micrococcus	<i>M. yunnanensis</i>	Endophytic, <i>Polyspora axillaris</i>	Zhao et al. (2009)
			<i>A. ilicis</i>	Pathogen, Holly bacterial blight of <i>Ilex opaca</i>	Young et al. (2004)
		Arthrobacter	<i>A. cupressi</i>	Rhizosphere soil of <i>Cupressus sempervirens</i>	Zhang et al. (2012)
			<i>K. palustris</i>	Rhizoplane of <i>Typha angustifolia</i>	Kovács et al. (1999)
		Kocuria	<i>K. rhizophila</i>	Rhizoplane of <i>Typha angustifolia</i>	Kovács et al. (1999)
			<i>M. azadirachtae</i>	Rhizoplane of <i>Neem (Azadirachta indica)</i>	Madhaiyan et al. (2010a)
		Microbacterium	<i>M. neimengense</i>	Rhizoplane of maize (<i>Zea mays</i>)	Gao et al. (2013)
			<i>M. foliorum</i>	Phyllosphere of grasses	Behrendt et al. (2001)
	Microbacteriaceae		<i>M. phyllosphaerae</i>	Phyllosphere of grasses	Behrendt et al. (2001)
			<i>A. bicolorata</i>	Narrow reed grass infected by the nematode <i>Heteroangina graminophila</i>	Evtushenko et al. (2001)
		Agreia	<i>A. versicolor</i>	Phyllosphere of potato	Behrendt et al. (2008)
			<i>A. albus</i>	Leaves and inflorescence of <i>Androsace</i> sp.	Dorofeeva et al. (2003)
		Agrococcus			
		Agromyces			

Table 3.1 (continued)

Order	Family	Main genera	Species	Plant	Reference
			<i>A. allii</i>	Rhizosphere soil of <i>Allium victorialis</i> <i>var. platyphyllum</i>	Jung et al. (2007)
			<i>A. braccium</i>	Rhizosphere of <i>Bruguera gymnorrhiza</i> (mangrove)	Takeuchi and Hatano (2001)
			<i>A. luteolus</i>	Surface of roots of <i>Sonneratia alba</i> (mangrove)	Takeuchi and Hatano (2001)
			<i>A. rhizospherae</i>	Rhizosphere of <i>Sonneratia alba</i> and <i>Bruguera gymnorrhiza</i> (mangrove)	Takeuchi and Hatano (2001)
		<i>Clavibacter</i>	<i>C. michiganensis sub sp.</i> <i>michiganensis</i>	Pathogen, Bacterial Canker of <i>Tomato</i> and <i>pepper</i>	Gartemann et al. (2003)
			<i>C. michiganensis sub sp.</i> <i>sepedonicus</i>	Pathogen, Potato (<i>Solanum tuberosum</i>)	Franc (1999)
			<i>C. michiganensis sub sp.</i> <i>nebraskense</i>	Pathogen, Gross's bacterial wilt and blight of <i>corn</i>	Jahr et al. (1999)
			<i>C. michiganensis sub sp. indosus</i>	Pathogen, Bacterial wilt and stunt of alfalfa (<i>Medicago sativa</i>)	Jahr et al. (1999)
		<i>Curtobacterium</i>	<i>C. flaccumfaciens pv. betae</i>	Pathogen, silencing disease of <i>red beet</i>	Collins and Jones (1983)
			<i>C. flaccumfaciens pv. beticola</i>	Pathogen, bacterial leaf spot of <i>sugar beet</i>	Chen et al. (2007)
			<i>C. flaccumfaciens pv. oortii</i>	Pathogen, bacterial wilt of <i>bean</i>	Collins and Jones (1983) Harding et al. 2007)
			<i>C. flaccumfaciens pv. oortii</i>	Pathogen, yellow pock of <i>tulip bulb</i>	Collins and Jones (1983)
			<i>C. flaccumfaciens pv. poinsettiae</i>	Pathogen, bacterial canker of common <i>poinsettia</i>	Collins and Jones (1983)

Table 3.1 (continued)

Order	Family	Main genera	Species	Plant	Reference
Propionibacteriales	Propionobacteriaceae	<i>Actinoplanes</i>	<i>A. missouriensis</i>	Endophytic, roots of lupin	El-Iarabily (2003)
		<i>Asanoa</i>	<i>A. hainanensis</i>	Rhizosphere of <i>Acrostichum spectiosum</i>	Xu et al. (2011)
		<i>Planosporangium</i>	<i>P. mesophilum</i>	Rhizosphere of <i>Betulla striata</i>	Cao et al. (2011)
		<i>Microlunatus</i>	<i>M. aurantiacus</i>	Rhizosphere of <i>Taxus chinensis</i>	Wang et al. (2008a)
		<i>Nocardioidea</i>	<i>N. caricicola</i>	Endophytic, <i>Carex scabrifolia</i>	Song et al. (2011)
	Nocacardioidaceae		<i>N. conyzicola</i>	Endophytic, roots of <i>Conyza Canadensis</i> (horse-weed)	Han et al. (2013)
			<i>N. endophyticus</i>	Endophytic, roots of <i>Artemisia princeps</i> (mugwort)	Han et al. (2013)
			<i>N. ultimimeridiana</i>	Rhizosphere of <i>Peucedanum japonicum</i>	Lee et al. (2011)
			<i>N. maradonensis</i>	Rhizosphere soil of <i>Peucedanum japonicum</i>	Lee et al. (2011)
			<i>N. perillae</i>	Endophytic, roots of <i>Perilla frutescens</i>	Du et al. (2013)
Pseudonocardiales	Actinopolymorpha	<i>A. pittoispori</i>		Endophytic, <i>Pittosporum phylliraeoides</i> (Australian native apricot tree)	Kaewkla and Franco (2011)
			<i>A. alba</i>	Rhizosphere of <i>Maytenus hookeri</i>	Cao et al. (2009)
			<i>A. cephalotaxi</i>	Rhizosphere of <i>Cephalotaxus fortunei</i>	Yuan et al. (2010)
			<i>K. amoyensis</i>	Rhizosphere of <i>Typhonium giganteum</i>	Xu et al. (2012b)
			<i>K. endophytica</i>	Endophytic, leaf of <i>Pittosporum phylliraeoides</i> (native apricot)	Kaewkla and Franco (2013a)
	Kribbella		<i>K. lupini</i>	Endophytic, roots of <i>Lupinus angustifolius</i>	Trujillo et al. (2006b)
			<i>K. solani</i>	Endophytic, <i>Solanum tuberosum</i> (potato tuber)	Song et al. (2004a)
			<i>P. adelaiddensis</i>	Endophytic, stem of Australian native <i>Eucalyptus microcarpa</i> (grey box tree)	Kaewkla and Franco (2010b)
			<i>P. artemisiae</i>	Endophytic, <i>Artemisia annua</i>	Zhao et al. (2011b)

Table 3.1 (continued)

Order	Family	Main genera	Species	Plant	Reference
Streptomycetales	Streptomycetaceae	Streptomyces	<i>P. eucalypti</i>	Endophytic, roots of <i>Eucalyptus</i>	Kaewkla and Franco (2010c)
			<i>P. endophytica</i>	Endophytic, <i>Lobelia clavata</i>	Chen et al. (2009a)
			<i>P. oroxyli</i>	Endophytic, roots of <i>Oroxylum indicum</i>	Gu et al. (2006)
			<i>Actinodalloteichus</i>	Rhizosphere of <i>Ficus religiosa</i> (fig tree)	Xiang et al. (2011)
			<i>Actinokineospora</i>	Rhizosphere of <i>Colocasia esculenta</i> (Elephant ear plant)	Intra et al. (2013)
			<i>Amycolata</i>	Root nodules and rhizospheres of <i>Alnus glutinosa</i> and <i>Alnus incana</i> (alder trees)	Evtushenko et al. (1989)
			<i>Amycolatopsis</i>	Endophytic, roots of <i>Samanea saman</i>	Duangmal et al. (2011)
			<i>A. ultimotia</i>	Rhizosphere of <i>Peucedanum japonicum</i>	Lee (2009a)
			<i>Saccharopolyspora</i>	Endophytes, roots of <i>Maytenus austroyunnanensis</i>	Qin et al. (2008a)
			<i>S. phatthalungensis</i>	Rhizosphere of <i>Hevea brasiliensis</i>	Duangmal et al. (2010)
			<i>S. tripterygii</i>	Endophytic, stem of <i>Tripterygium hypoglaucum</i>	Li et al. (2009)
			<i>S. alni</i>	Endophytic, <i>Alnus nepalensis</i>	Liu et al. (2009)
			<i>S. aureofaciens</i>	Pathogen, russet scab disease of Potato	Faucher et al. (1993)
			<i>S. cavarescibies</i>	Pathogen, potato scab	Goyer et al. (1996)
			<i>S. lacticiproducens</i>	Rhizosphere of tomato	Zhu et al. (2011)
			<i>S. marookonensis</i>	Rhizosphere of <i>Arganis spinosa</i>	Bouizgame et al. (2009)
			<i>S. ipomea</i>	Pathogen, <i>Sweet potato (Ipomea batata)</i> soil rot	Clark et al. (1998)
			<i>S. reticuliscabies</i>	Pathogen, netted scab disease of Potato	Bouchek-Mechiche et al. (2000)
			<i>S. scabies</i>	Pathogen, common scab disease of Potato	Leiminger et al. (2013)

Table 3.1 (continued)

Order	Family	Main genera	Species	Plant	Reference
Streptosporangiales		<i>Kitasatospora</i>	<i>S. turgidiscabies</i>	Pathogen, common scab disease of Potato	Leiminger et al. (2013)
			<i>K. arboriphila</i>	Soil from the roots of <i>Maytenus ilicifolia</i>	Groth et al. (2004)
			<i>K. paranensis</i>	Rhizosphere of <i>Maytenus ilicifolia</i>	Groth et al. (2004)
			<i>K. putterlickiae</i>	Rhizosphere soil of <i>Putterlickia verrucosa</i>	Groth et al. (2003)
			<i>K. terrestris</i>	Soil from the roots of <i>Maytenus aquifolia</i>	Groth et al. (2004)
			<i>M. hainanensis</i>	Rhizosphere of <i>Excoecaria agallocha</i>	Xu et al. (2012a)
			<i>N. antimicrobica</i>	Endophytic, leaves of <i>Maytenus austroyunnanensis</i>	Qin et al. (2009c)
			<i>N. endophytica</i>	Endophytic, <i>Artemisia annua</i>	Li et al. (2011)
			<i>N. rhizophila</i>	Rhizosphere of <i>Tripterygium wilfordii</i> (perennial vine)	Zhao et al. (2011a)
			<i>N. wenchangensis</i>	Rhizosphere of <i>Bruguiera sexangula</i> (mangrove)	Wang et al. (2011b)
Thermomonosporaceae		<i>Nocardiopsis</i> <i>Actinomadura</i>	<i>N. tropica</i>	Rhizosphere of <i>Casuarina</i> sp.	Evtushenko et al. (2000b)
			<i>A. flavalba</i>	Endophytic, leaves of <i>Maytenus austroyunnanensis</i>	Qin et al. (2009d)

^a The Orders *Actinomycetales*, *Actinopolysporales* and *Bifidobacteriales* are not represented as to our knowledge, not reported as associated to plants. For the orders that figure in the table, not all families and genera are represented. For some species frequently encountered in the rhizosphere e.g. *Streptomyces*, only some species are presented. Most of these species were identified by polyphasic approach (phenotypic, chemotaxonomic and genotypic methods)

(Gonzalez-Franco et al. 2009). The use of culture independent techniques based on phospholipid fatty acid analysis (PLFA) and analysis of nucleic acids: pyrosequencing (Uroz et al. 2010), TGGG fingerprints of 16S rDNA (Gomes et al. 2001), DGGE of 16S rRNA and 16S rDNA (Duineveld et al. 2001) clone library method (McCaig et al. 1999) and rhizosphere 16S rRNA microarray (Mendes et al. 2011) and from soil has allowed to determine their diversity and their unculturable fraction.

Actinobacteria represents a large fraction of microbial populations in the root systems (Miller et al. 1989; Crawford et al. 1993; McCaig et al. 1999; Gomes et al. 2001) and is well established that they are dominant fraction of the microbial community in soils of wild and agricultural plant species (Miller et al. 1989; Pandey and Palni 2007; Jayasinghe and Parkinson 2007). Together with other phyla, they account for a large proportion in the rhizosphere of numerous plants including Oak (*Quercus* sp.) (Uroz et al. 2010), potato (*Solanum tuberosum*) (Weinert et al. 2011), sugar beet (*Beta vulgaris*) (Mendes et al. 2011), rice (*Oryza sativa*) (Knief et al. 2012) and thale cress (*Arabidopsis thaliana*) (Bulgarelli et al. 2012; Lundberg et al. 2012). Bacterial 16S rRNA gene pyrosequencing revealed members of the phyla Actinobacteria and α -Proteobacteria as dominant taxa in both the unplanted soil and rhizosphere. 16S rDNA derived clone libraries of rhizosphere and rhizoplane bacterial community structure in oilseed rape (*Brassica napus*) (Macrae et al. 2000; Kaiser et al. 2001) allowed to detect abundance of α -Proteobacteria higher than Actinobacteria. Similar results were found by McCaig et al. (1999) in grass rhizospheres where Actinobacteria were found to be the second-most-abundant group after the α -Proteobacteria. However, Actinobacteria were found as dominant in the rhizosphere of numerous plants including barley (Yang and Crowley 2000), maize (Gomes et al. 2001), Alfalfa and *Chenopodium album* (Schwieger and Tebbe 2000), sagebrush (*Artemisia tridentata*) (Gonzalez-Franco et al. 2009), clover and ryegrass (Sperber and Rovira 1959) and *Chrysanthemum* (Duineveld et al. 2001). Gonzalez-Franco et al. (2009) showed by PCR-DGGE fingerprinting performed on rhizosphere DNA that an actinomycete profile represents the dominant fractions.

One major trait of the rhizosphere effect is the differential abundance of bacteria and fungi between rhizosphere and bulk soils. In addition, contrasted bacterial diversity between rhizosphere and non rhizosphere soil was reported (Uroz et al. 2010). In numerous studies, Actinobacteria were found to be present at higher amounts in rhizospheres compared to bulk soil (Smalla et al. 2001). The rhizosphere/bulk soil ratio for Actinobacteria ranges from 5 to 10 (Morgan et al. 2005). The rhizosphere exerts its effect on activity and composition of microbial communities via root exudates (rhizosphere effect) (Grayston 2000). The rhizosphere effect could lead to plant species-specific microflora (Grayston et al. 1998; Smalla et al. 2001; Kowalchuk et al. 2002) and is likely to be due to differences in root exudation and rhizodeposition. The influence of plant species on bacterial root colonization was demonstrated for example by Smalla et al. (2001) by using PCR-DGGE fingerprints of the bacterial communities in the rhizosphere of three crops: potato, strawberry, and oilseed rape. Earlier, Kortemaa et al. (1994, 1997) used SEM to assess the distribution of *Streptomyces griseoviridis* in rhizosphere of two plants. These authors showed that *S. griseoviridis* colonize more efficiently

root-hair zone in the rhizosphere of turnip than those of carrot. In addition, differences in their diversity and counts were found between rhizosphere and rhizoplane. Another example where proportion of Actinobacteria in the rhizosphere was found to be higher than in the rhizoplane was reported in three tree species: hybrid larch (*Larix eurolepis*), Sitka spruce (*Picea sitchensis*) and sycamore (*Acer pseudoplatanus*). This behavior contrasted with that of other bacteria and difference was attributed to differences in exudates composition in the two areas. It was concluded that Actinomycetes tend to grow on complex polymers in rhizospheric soil, whereas other bacteria prefer simple, readily utilizable carbon sources in the rhizoplane (Grayston 2000).

In the last decades, interests in the role of the beneficial bacterial interactions within the plant rhizosphere for crop production enhancement and disease management have increased (Mendes et al. 2011). Actinobacteria could play an important role in plant health by acting as plant growth promoting rhizobacteria (Hamdali et al. 2008; Hasegawa et al. 2008; de Vasconcellos and Cardoso 2009) and as the main microorganisms implicated in controlling the infection of roots by soil-borne pathogenic fungi and bacteria (Williams et al. 1989, Hamby 2000, El-Tarabily and Sivasithamparam 2006). Plant growth promoting rhizobacterial (PGPR) effects rely essentially on their ability to solubilize phosphate (El-Tarabily et al. 2008) or to produce phytohormones (El-Tarabily 2008; Hamdali et al. 2008). As root aggressive colonizers, they could also compete with other telluric microorganisms, thus act as excellent biological control agents. *Streptomyces* have been implicated in naturally-occurring and induced disease suppressive soils to fungal phytopathogens (Weller et al. 2002; Hjort et al. 2010; Mendes et al. 2011). Mendes et al. (2011) by using 16S rRNA microarray found that α -Proteobacteria played a prominent role in soil suppression of *Rhizoctonia solani*. Actinobacteria largely accounted for the differentiation between suppressive and conducive soil. In addition, sustainability of the efficiency of Actinobacteria to control plant disease is mainly due by their great adaptability to soil conditions where they accomplish various complex interactions with plant roots and other microorganism. By reducing phytopathogens population's number and or their aggressiveness, they contribute to the crop protection. Actinobacteria display diverse modes of action towards soil borne plant pathogenic fungal populations. These modes of actions implicated in biocontrol are: competition that rely for example on their ability to produce siderophore that chelate iron ions in soils (Macagnan et al. 2008; Khamna et al. 2009) antibiosis (Berdy 2005) and parasitism by chitinolytic and glucanolytic enzymes (Valois et al. 1996; Joo 2005; El-Tarabily and Sivasithamparam 2006). It has been suggested that competitive antibiotic-producing organisms have advantage over nonproducing microbes in soil. Particularly, Actinobacteria producing antibiotics possess a moderating effect on other microbial population. They could act as Mycorrhiza helpers but also as fungi antagonists. *Streptomyces* sp. AcH 505 isolated from the rhizosphere of a Norway spruce (*Picea abies*) stand significantly promoted the mycelial growth and mycorrhization rate of the symbiotic fungus *Amanita muscaria*. This effect is due to the production of IAA like growth promoter; auxofuran (Riedlinger et al. 2006).

3.3.2 Endophytic Actinobacteria

Like all endophytes endophytic Actinobacteria colonize parts inside of plants. Hallmann et al. (1997) defined endophyte bacteria as microorganisms that do not visibly harm the plant and can be isolated from surface disinfected plant tissues or extracted from inside the plant.

The first report on endophytic Actinobacteria was published by Hasegawa et al. (1978) who described an *Actinosynnema* from a grass blade. Thereafter, endophytic Actinobacteria were detected in many other plants including both herbaceous and ligneous plants of diverse species (Sardi et al. 1992; El-Shatoury et al. 2013). Several workers showed that they coexist with other microorganisms forming endophytic microbial community (Sessitsch et al. 2002) for which diversity in general could be influenced by plant nature and even cultivars within the same species (Sessitsch et al. 2002). In general, studies of the diversity among endophytic bacteria allowed concluding that endophytic Actinobacteria are among dominant endophytic phyla inside plants. Examples of plants where Actinobacteria represent a proportion of endophytic microbiota are *Solanum tuberosum* (Sessitsch et al. 2002; Manter et al. 2010), *Oryza sativa* (Sessitsch et al. 2012) and *Arabidopsis thaliana* (Bulgarelli et al. 2012).

Visualization of endophytic Actinobacteria was performed by using light microscopy (Okazaki 2003), SEM (Sardi et al. 1992; Franco et al. 2007), TEM (Suzuki et al. 2005) and confocal electron microscopy (CLM) (Franco et al. 2007; Coombs and Franco 2003b). Sardi et al. (1992) observed Actinobacterial endophytes localized in cortical tissues of tomato roots. El-Tarabily et al. (2009) localized sporangia of *Actinoplanes* within cells of the cucumber root cortex. In wheat, Okazaki (2003) observed *Microbispora* on the surface and inside of epidermal cells. Coombs and Franco (2003b), by using CLM microscopy, observed microcolonies of a *Streptomyces* sp. strain EN27 previously tagged with the *gfp* gene that develop intracellularly in endosperm and in emerging embryo radicals of wheat, generally in close proximity to the cell walls. Thereafter, Franco et al. (2007) by using CLM confirmed that GFP-tagged *Streptomyces* sp. EN27 sporulated inside the epidermal cells of inoculated wheat seedlings. No cortical colonization of wheat root was observed in this investigation. All these observations may provide a better insight into the intracellular lifestyle of root endophytic Actinobacteria. In leaves of *Rhododendron*, *S. galbus* hyphae were observed individually or in colonies in intercellular spaces between epidermal and mesophyll cells but not inside those cells (Suzuki et al. 2005). A particular trait of leaf colonization is that hyphae were observed to be embedded in an amorphous, mucilage-like material (Sardi et al. 1992; Minamiyama et al. 2003; Suzuki et al. 2005; Shimizu et al. 2009). Enzymatic activities were found to be associated with such trait (Minamiyama et al. 2003; Suzuki et al. 2005). These observations encouraged Shimizu (2011) to speculate that endophytic actinomycetes adhere firmly to the host plant surfaces and acquire the nutrition by using extracellular polymers containing adhesive compounds and hydrolytic enzymes, similar to that of fungi.

However, the in situ role of endophytic Actinobacteria to the plant life is relative poorly understood although some works reported relationship between their presence and resistance to various biotic and abiotic stress such as light deficiency, diseases (Sessitsch et al. 2002), drought (Hasegawa et al. 2004) or as PGP that produce IAA (El-Tarabily et al. 2009; Ghodhbane-Gtari et al. 2010; Nimnoi et al. 2010), pteridic acids (Igarashi et al. 2002) or indole-3-pyruvic acid (IPYA) (El-Tarabily et al. 2009). Association of endophytic Actinobacteria with *Rhizobium-legume* symbionts was also reported. Tokala et al. (2002) reported that root nodules of *Pisum sativum* taken from agricultural fields in the Palouse hills of northern Idaho were found to be colonized by actinomycete hyphae. They showed that a *Streptomyces* was responsible of an increase in nodulation frequency in *Pisum sativum*. They also showed in *Streptomyces lydicus* colonized Pea roots, an increased nodulation frequency by *Rhizobium* spp. and nitrogenase activity, possibly at the level of infection by *Rhizobium* sp. This could support the hypothesis that root and nodule colonization is one of several mechanisms by which *Streptomyces* acts as a naturally occurring PGPB in pea. Moreover, Trujillo et al. (2010) hypothesized that endophytic *Micromonospora* populations are natural inhabitants of nitrogen-fixing root nodules of *Lupinus angustifolius* and that they also fix nitrogen in symbiosis with their host. These kinds of associations are thought to be spread in leguminous plants (Tokala et al. 2002; Trujillo et al. 2010). Actinobacteria could also play a role related to the health of plants (Shimizu 2011). Tian et al. (2004), found that *Streptomyces griseofuscus* is the commonest population of endophytic actinomycetes, and constituted the greatest part of all the antagonistic communities. Population of endophytic actinomycetes including *S. griseofuscus* and *S. hygroscopicus* were found to be antagonistic to the rice pathogens *Magnaporthe grisea*, *R. solani*, *Xanthomonas oryzae* pv. *oryzae* and *F. moniliforme*. Like most rhizospheric Actinobacteria, endophytic actinomycetes were found to produce active antagonistic metabolites in plant tissues, including antibiotics, enzymes and siderophores (Trejo-Estrada et al. 1998). Actinobacteria with in vitro antipathogenic activities were isolated from rice (Tian et al. 2004), wheat (Coombs and Franco 2004) and banana (Cao et al. 2004a). Due to their ability to colonize the internal tissues of the host plant and to be well adapted to this microcosm, endophytic Actinobacteria exploited as biocontrol agents of plant diseases in field soils. Biocontrol assays include control of *Fusarium* wilt disease on cotton (Chen et al. 1995), take-all disease in wheat (*Triticum aestivum*) caused by *Gaeumannomyces graminis* var. *tritici* (Coombs and Franco 2004), root rot of lupin caused by *Plectosporium tabacinum* (El-Tarabily 2003), damping-off and root and crown rots caused by *Pythium aphanidermatum* in cucumber (*Cucumis sativus* L.) (El-Tarabily et al. 2009), in chickpea (*Cicer arietinum* L.) root rot caused by *Phytophthora medicaginis* (Misk and Franco 2011) and many other plant diseases. In this context, it could be suggested to isolate them from healthy plants and inoculate them to plants of the same species or related species. Work by Sessitsch et al. (2002) found that a higher abundance and diversity of *Streptomyces scabiei*-related species was found in tubers of the variety Mehlige Mühlviertler, known for its resistance against potato common scab than susceptible varieties (Achirana Inta and Bionta). It may suggest that disease resistance could be

linked to such significant functional biodiversity. In addition, root inoculated Actinobacteria could also lead to disease resistance enhancement in host plants (Kunoh 2002; Lehr et al. 2008)

In wheat, Coombs and Franco (2003b) showed that GFP-expressing endophytic *Streptomyces* sp. could colonize seed embryos and emerging radicals. Colonization occurred rapidly and the strain could be visualized in the growing plant from the first day suggesting its interaction with plants at early stages. Thus, Actinobacteria could most likely to be isolated from young and older plants. Isolation of endophytic Actinobacteria is mainly performed by cultivation of surface-sterilized roots or leaves on appropriate media (Sardi et al. 1992; Coombs and Franco 2003a, b). Their enumeration and characterization could be performed by culture methods (El-Tarabily 2003; El-Tarabily et al. 2009). Most frequently isolated endophytic Actinobacteria belong to the genera *Streptomyces* (Coombs and Franco 2003a; Taechowisan et al. 2003; Shimizu et al. 2009; Nimnoi et al. 2010), *Micromonospora* (Coombs and Franco 2003a; Lee et al. 2008; Taechowisan et al. 2003), *Microbispora* (Okazaki 2003; Taechowisan et al. 2003; Coombs and Franco 2003a, 2004; Lee et al. 2008), *Microtetraspora* (de Araújo et al. 2000; El-Shatoury et al. 2013), *Nocardia* (Taechowisan et al. 2003; Cao et al. 2004a, b), *Nocardioides* (Coombs and Franco 2003a; Coombs et al. 2004; Tian et al. 2007) and *Streptosporangium* (de Araújo et al. 2000). Works aiming to isolate endophytic Actinobacteria from the endosphere of various plants showed that *Streptomyces* species are the most dominant species (Taechowisan et al. 2003; Coombs and Franco 2003a; Sessitsch and Berg 2004; Tian et al. 2007; Janso and Carter 2010; El-Shatoury et al. 2013). For example, Taechowisan et al. (2003) isolated 330 endophytic *Actinomycetes* isolates of 31 species from plants, the majority of these strains were *Streptomyces* and the remainder were *Microbispora*, *Nocardia* and *Micromonospora*. Tian et al. (2004) studied the diversity of endophytic fungi and actinomycetes populations from four rice cultivars and found that preponderant endophytic actinomycetes belonged to *Streptomyces* with an incidence of *S. griseofuscus* ranged from 36.1 to 69% out of all the different rice cultivars. Endophytic *Streptomyces* was found to be the most abundant actinobacterium in roots of many dicotyledons including tomato (*Lycopersicon esculentum*) (Sardi et al. 1992; Cao et al. 2004b) and eaglewood (*Aquilaria crassna*) (Nimnoi et al. 2010). Some monocotyledons such as wheat (Coombs and Franco 2003a), rice (*Oryza sativa*) (Tian et al. 2007) and banana (*Musa acuminata*) (Cao et al. 2004a, b) have reported monogenus existence *Streptomyces* (Sessitsch et al. 2002). In addition to roots, Actinobacteria could also colonize plant leaves (Kizuka et al. 1998; Shimizu et al. 2000; Okazaki 2003; Cao et al. 2004a; Suzuki et al. 2005). *S. galvus* was found in the leaves of tissue-cultured *Rhododendron* (Suzuki et al. 2005). However, in other plants, *Microbispora* was found to be prevalent in comparison with *Streptomyces* (Okazaki 2003). Dominance of *Microbispora* was reported for example in Maize (*Zea mays*) (de Araújo et al. 2000) and Chinese cabbage (*Brassica campestris*) (Lee et al. 2008).

Molecular methods allow detecting a great diversity within plants. Garbeva et al. (2001) and Conn and Franco (2004) stated that culture-independent methods revealed a higher endophytic diversity than that obtained by isolation and culture

dependant methods. Tian et al. (2007) analyzed the 16S rRNA genes of numerous endophytic Actinobacteria and found both known and uncultured (unidentified) Actinobacteria among the clones of root and stem origin of paddy. The molecular studies on endophytic bacterial diversity could reveal a large richness of species (Rosenblueth and Martinez-Romero 2006; Kaewkla and Franco (2013b). For example, by using 16S rRNA gene analysis, an extremely rich diversity was detected in medicinal plants from the tropical rain forest in china. In this Study, rare endophytic Actinobacteria belonging to genera *Saccharopolyspora*, *Dietzia*, *Blastococcus*, *Dactylosporangium*, *Promicromonospora*, *Oerskovia*, *Actinocorallia*, and *Jiangella* were identified (Qin et al. 2009b). New endophytes species *Microtetraspora*, and *Intrasporangium*, which have not been previously reported to be endophytic were detected by using rRNA gene sequences (El-Shatoury et al. 2013). Also, recently published works revealed the endophytic ability of *Williamsia* (Kaewkla and Franco 2013b; Fang et al. 2013). By T-RFLP, Conn and Franco (2004) found a relationship between the diversity of endophytic Actinobacteria in wheat roots. A comparison of endophytic and rhizosphere microbial communities confirmed that endophytes bacterial populations represent a subset of rhizosphere bacteria (Sturz et al. 1997; Germida et al. 1998). It was hypothesized that root endophytic bacteria could be originated from the rhizosphere (Hallmann et al. 1997). Thus, endophytic predominance of *Streptomyces* could probably be due to the prevalence of this species in soils surrounding roots in comparison with species from other Actinobacteria genera. Their prevalence in a wide range of plant species could be also due to their high versatility as endophytes are largely influenced by the nature of plant chemical constituents.

3.3.3 *Symbiotic Actinobacteria: Genus Frankia*

The name of the genus *Frankia* was first proposed by Brunchorst (1886–1888). The Order *Frankiales* comprises the monogeneric family *Frankiaceae* and other families. The cell-wall diamino acid is meso diaminopimelate (meso-DAP) in all families except for the family *Sporichthyaceae*, which contains LL diaminopimelate. The family *Frankiaceae* comprises only the genus *Frankia* (Normand et al. 1996) which is the unique actinobacterium that could form N₂-fixing root nodule symbioses with numerous phanerogammes. These plants are called actinorhizal plants. However, *Frankia* is facultative symbionts and could live separately in saprophyte form (Normand et al. 2007), as a proportion of *Frankia* with infective capacity can survive in soils that are devoid of host plants (Smolander and Sundman 1987; Maunuksela et al. 1999). In laboratory, *Frankia* growth is a time consuming operation and need special procedures (Baker 1988). Frequently, long periods (10 to 20 days) are required for their cultivation but reduce the period by supplementing with flavonoid quercetin (Sayed and Wheeler 1999). This period was reduced to three days by using gellan gum as a gelling agent and peptones (Bassi and Benson 2007). Currently, no species names are adopted and only strain designations are available. All

available strains are represented as the name of the Genus followed by an isolate number, *Frankia* does not form aerial mycelium, possess chemotype III (meso-DAP) and is characterized by the differentiation of specialized structures named vesicles measuring 2 and 6 μm in diameter which are the sites of nitrogen fixation. In addition, the walls of these structures are composed of lipid layers which act as a barrier to ambient oxygen levels, and permit to avoid the inhibition of nitrogenase activity. Actinorhizal plants comprise 200 plant species belonging to 25 genera of woody plants from eight non-leguminous dicotyledonous angiosperm families *Casuarinaceae*, *Betulaceae*, *Rhamnaceae*, *Rosaceae*, *Myricaceae*, *Coriariaceae*, *Elaeagnaceae* and *Datisceae* (Benson and Clawson 2000; Pawlowski and Sirenberg 2003; Chaia et al. 2010). They are considered as early successional species following deglaciation (Chapin et al. 1994) or fires (Roy et al. 2007) and thus be used for conditioning soils for revegetation (Roy et al. 2007).

Enumeration of *Frankia* has been performed either by nodule formation method after inoculation (Huss-Danell and Myrold 1994; Maunuksela et al. 1999) as by molecular methods (Myrold et al. 1994; Normand et al. 1996; Mirza et al. 2009a). Plant bioassay is one of the simplest methods to quantify *Frankia* in soil by targeting infective *Frankia* population by inoculations to a specific host (trap plant). Nodulation unit (nu) per gram or per cm^3 soil is determined by nodulation capacity (NC) which is expressed as the number of *Frankia* particles per unit of soil, each of numbered *Frankia* induces one nodule in the nodulation test. Nodulation could also be determined by the MPN enumeration technique in which host plants are inoculated with serial dilutions of *Frankia* containing samples and formed nodules are enumerated (Huss-Danell and Myrold 1994; Myrold et al. 1994; Chaia et al. 2006). Estimates by MPN were found to be in agreement with NC technique, especially between 30 and 300 nu g^{-1} soil, for *Alnus* infective *Frankia* (Huss-Danell and Myrold 1994). By these methods, nodulation units between 0 and 4600 g^{-1} soil have been obtained for different soils (Myrold et al. 1994).

However, the bioassay methods are of limited value as nodulation could be the result of different propagules. i.e., spore, hyphae or even a whole colony. It was established by genotypy that the fraction of infective population represents only a small portion of the total population of *Frankia* present in soil (Picard et al. 1992; Myrold et al. 1994; Zepp et al. 1997b; Maunuksela et al. 1999). Recent genotypic analyses revealed differences between detectable *Frankia* populations in soil and those in root nodules indicating the inadequacy of bioassays for the analysis of *Frankiae* in soil and the role of plants in the selection of *Frankiae* from soil for root nodule formation (Mirza et al. 2009a). Since only nodule forming populations on a specific host plant are detected, less genetic information on *Frankia* in soil are available in comparison with root nodules. Thus, genomic enumeration methods proved to have advantages over plant bioassays in assessing soil *Frankia* populations. Oligonucleotide probes targeting rRNA was the first molecular method used to detect and quantify *Frankia* in soil (Hahn et al. 1990). Direct DNA extraction followed by either nested PCR (Myrold et al. 1994) or booster PCR (Picard et al. 1992) amplification combined to MPN is also used. These methods of enumeration allowed estimating genomic units (gu) per g or per cm^3 of soil (a genomic unit

correspond to amount of *Frankia* containing a single genome). All these methods allowed a detection limit of 10^4 unit g^{-1} soil. In situ hybridization was also used to detect and quantify *Frankia* in the environment. Epifluorescence microscopy observation of *Frankia* was assessed by in situ hybridization with specific labeled probes that targets rRNA (Hahn et al. 1997) or specific sequences on the 23S rRNA insertion for *Frankia* (Hahn et al. 1997; Zepp et al. 1997a; Maunuksela et al. 1999; Mirza et al. 2007).

It is interesting to note that *Frankia* species were divided into groups according to the species of the associated woody dicotyledons involved in the association: Group 1: strains infective on *Alnus* (Betulaceae) and *Myrica* (Myricaceae), Group 2: strains infective on *Casuarina* (Casuarinaceae) and *Myrica* (Myricaceae), Group 3: strains infective on *Myrica* (Myricaceae) and *Elaeagnus* (Elaeagnaceae) and Group 4: only members of Elaeagnaceae (Baker 1987; Torrey 1990). Dobritsa (1998) used the following phenotypic traits to phenotypically cluster *Frankia* strains: susceptibility to antibiotics, pigment production and host specificity and found concordance with this grouping. FAMES analysis by Simon et al. (1989) corroborated this taxonomy. These subdivisions were also supported by results of DNA–DNA relatedness among *Frankia* strains (Fernandez et al. 1989; Lumini et al. 1996). Within host infection groups, relatedness ratios between 67 and 94% were observed, and levels lower than 50% were found between different host infection groups (Fernandez et al. 1989; Akimov and Dobritsa 1992; Lumini et al. 1996). A concordance between phenotypic clustering and *Frankia* genospecies according to DNA hybridization data was reported by Dobritsa (1998), confirming that genotype reflects phenotype within the genus *Frankia*. Genomic approaches by 16S ribosomal DNA partial or complete sequencing are powerful tool to differentiate *Frankia* strains (Nazaret et al. 1991; Niner et al. 1996; Benson et al. 1996; Clawson and Benson 1999; Clawson et al. 2004; Valdés et al. 2005; Carro et al. 2013). Phylogenetic relationships among *Frankia* strains were investigated in relation to host specificity by complete sequencing of 16S rDNA. According to 16S rDNA phylogeny, *Casuarina*-specific strains were found to be more closely related to *Alnus*-specific strains and results allowed to group *Frankia* strains within new subdivisions of host infection groups. Four genotypic clusters were thus described (Normand et al. 1996). These host infection groups are (i) *Frankia alni* and other typical nitrogen-fixing strains belonging to the *Alnus* and the *Casuarina* host infection groups, respectively, (ii) uncultured endophytes of *Dryas*, *Coriaria* and *Datisca* species, (iii) strains of the *Elaeagnus* (Elaeagnaceae) host infection group and (iv) atypical non-nitrogen-fixing strains. Sequences similarities of the actinomycetes-specific insertion in domain III of the 23S rRNA was also used and served to confirm the 16S-rRNA sequence classification of the strains into these four host-infection groups (Hönerlage et al. 1994; Maunuksela et al. 1999).

It was established by Normand et al. (1988) that *Frankia* strains showed conservation of sequences of the functional genes *nif* involved in nitrogen fixation (nitrogenase reductase). Gene sequences of such functional genes represent a strong tool to assess diversity and evolutionary divergence of *Frankia* strains. Within this framework, the following genes were successfully targeted for the characterization

of isolates as well as for the identification of uncultured endophytes in root nodules: IGS spacer between the *nifD* and *nifK* genes (*nifD*-K) (Navarro et al. 1997; Dai et al. 2004) the Fe-protein *nifH* combined to *nifH* PCR-RFLP (Gtari et al. 2007) and glutamine synthetase (*glnA*) gene sequences combined to 16S rDNA sequences (Clawson et al. 2004). Though *nifH* gene is not a perfect target for the quantification of *Frankia*, this technique allowed a detection of cell densities of up to 10^6 cells g^{-1} soil (Samant et al. 2012). Work by Gtari et al. (2007) showed that *nifH* gene sequences showed consistency with 16S rRNA and GlnII phylogenetic trees. In addition, sequence similarity values of 93–97% for the *nifH* gene fragments allowed confirming assignments of *Frankia* strains to the above-mentioned genomic groups (Mirza et al. 2009a). In addition, *nifH* gene confirmed the existence of genomic groups (genospecies) within the host infection groups *Alnus/Casuarina* (cluster 1) and *Elaeagnus* (cluster 3) (Fernandez et al. 1989; Mirza et al. 2009a). Also, fingerprinting including PCR-RFLP of 16S rRNA gene and particularly those encoding functional activities (Normand and Bousquet 1989; McEwan et al. 1994; Hahn et al. 1999; Lumini and Bosco 1999; Cournoyer and Lavire 1999; Huguet et al. 2001) are also of great interest. Diversity of *Frankia* strains in culture and within root nodules was assessed by PCR-RFLP of the intergenic spacer (IGS) between ribosomal 16S–23S subunits (IGS 16S–23S) (Rouvier et al. 1996; Hahn et al. 1999) between *nifD* and *nifK* genes (IGS *nifD*-K operon) (Jamann et al. 1993; Hahn et al. 1999; Dai et al. 2004) or between *nifH* and *nifD* (IGS *nifH*-D) genes (Jamann et al. 1993; Hahn et al. 1999). Fingerprinting targeted also genes such as glutamine synthetase II (GlnII) (Cournoyer and Lavire 1999; Hahn et al. 1999; Navarro et al. 1999).

Several works focused on comparison of the diversity of *Frankia* in different actinorhizal plants growing in different geographic areas (Normand et al. 1996) and in some cases from sources as distant as continents (Mirza et al. 2009b). Both phylogenetic (Normand et al. 1996) and DNA–DNA hybridization (Akimov and Dobritsa 1992) data have shown that major groups of *Frankia* strains are reflected by the plant origin (biogeography) and/or host specificity. Using *glnII*, phylogenetic relationships within the genus led to the conclusion that *Frankia* diversification seems to be related to their ability to infect actinorhizal plants (Cournoyer and Lavire 1999). The sequencing of whole genomes of *Frankia* strains (Normand et al. 2007) is promising. Sequencing of three *Frankia* whole genomes could contribute to obtain more genome informations. Normand et al. (2007) showed that genomes of these closely related strains (97.8–98.9% identity of 16S rRNA genes) revealed variable sizes from 5.43 to 9.04 Mbp and stated that genome expansions as well as reductions could be related to biogeographic history of the symbiosis and host plant speciation. This work could be extended to all described strains to assess the evolutionary relationships and ecology of *Frankia*. Other recent methods based on the sequencing and comparison of specific housekeeping genes such as the *recA* gene (Maréchal et al. 2000) and *rpoB* and *rpoD* genes (Bernèche-D'Amours et al. 2011) could be a powerful tool to assess diversity and functionality of *Frankia* strains and to accurately characterizing the uncultured populations that are present in soils and nodules. MALDI-TOF MS method had also demon-

strated its usefulness as a rapid and reliable tool for the characterization of *Frankia* strains (Hahn et al. 2011). In addition, it allowed assigning *Frankia* strains to their respective host infection groups and showed consistency with their classification into genomic sub-groups based on comparative sequence analysis of *nifH* gene fragments.

The issue of evolutionary origin of *Frankia* was questioned by some research works. *Geodermatophilus* belonging to the family Geodermatophilaceae under the same order as *Frankia* (Frankiales) was first thought to be the closest neighbour of *Frankia* as the two organisms share morphological features (mainly the presence of multilocular sporangia) (Hahn et al. 1999). However, 16S rRNA allows concluding that *Frankia* is phylogenetically close to *Acidothermus cellulolyticus* from the monogeneric family Acidothermaceae (Order Frankiales) (Normand et al. 1996). The close proximity of *Frankia* to *Acidothermus* was lately confirmed by analyses of *recA* housekeeping gene (Maréchal et al. 2000). Gtari et al. (2007) revealed new diazotrophic species with intermediate taxonomic position between *Frankia* and *Micromonospora* according to the RFLP and sequences of the *nifH* gene. In addition, Carro et al. (2013) found that *Micromonospora* strains are ubiquitous in actinorhizal nodules and *Micromonospora* strains with nitrogenase activity were isolated from nitrogen-fixing root nodules of actinorhizal plants (Niner et al. 1996; Valdés et al. 2005; Trujillo et al. 2006a). This work and others demonstrated the existence in root nodules of non-*Frankia* symbionts that harbors nitrogenase gene and fix nitrogen (Valdés et al. 2005; Gtari et al. 2007; Ghodhbane-Gtari et al. 2010). Carro et al. (2013) stated that *Micromonospora* isolates are normal occupant of actinorhizal nodules but their function is unknown at the moment. In addition, new *Micromonospora* species were also discovered in nodules of *P. sativum* (Carro et al. 2012). The involvement of *Micromonospora* in plant-microbe associations especially their plausible function as nitrogen fixing symbionts still poorly understood. Phenotypically, *Micromonospora* is characterized by filaments diameter thinner ($<0.5\ \mu\text{m}$) than *Frankia* ($0.5\text{--}2\ \mu\text{m}$) and as for *Frankia* do not develop aerial mycelia, *Micromonospora* cell wall contains peptidoglycan of the meso-DAP type and/or its 3-hydroxy derivative, and glycine. It also contains arabinose and xylose (chemotype II) (Holt et al. 2000). Generally, *Micromonospora* does not develop nitrogen-fixing vesicles typically found in *Frankia*. *Frankia* possess the type III cell wall containing meso-DAP (Lechevalier and Lechevalier 1990), alanine, glutamic acid, muramic acid, and glucosamine. Phylogenetic analysis based on 16S rRNA gene sequences and other genomic analyses were used to differentiate *Frankia* and *Micromonospora* in several studies (Niner et al. 1996; Valdés et al. 2005; Trujillo et al. 2006a; Carro et al. 2013). Gtari et al. (2007) showed that a high conservation level of *nifH* sequences has been detected between *Frankia* strains and the intermediate strain for which a horizontal transfer of the *nif* genome is not excluded. Also, though phenotypic differences, *Micromonospora* strains for which partial *nifH*-like gene sequences of 99% similarity with those of the *nifH* gene from *Frankia alni* ACN14a were isolated from nodules of a legume (Trujillo et al. 2010). These results suggest that these two species probably had a common Actinobacterial ancestor (Gtari et al. 2007).

3.3.4 Plant Pathogenic Actinobacteria

Study of the diversity of plant pathogenic bacteria has a primordial interest especially for disease epidemiology, such as studies on the source and outbreaks of the disease (Mogen et al. 1990; de Leon et al. 2009; Baysal et al. 2010) or on ways to prevent the spread of the pathogen. Plant pathogenic Actinobacteria include species belonging to the following orders and families:

A—Order Micrococcales:

- Micrococcaceae: one genus: *Arthrobacter* (Collins et al. 1981).
- Microbacteriaceae: four genera *Curtobacterium* (Goodfellow 1984), *Clavibacter* (Eichenlaub and Gartemann 2011), *Leifsonia* (Davis et al. 1984) and *Rathayibacter* (Zgurskaya et al. 1993).

B—Order Corynebacteriales, Family Nocardiaceae: one genus, *Rhodococcus* (Goodfellow 1984).

All these genera are non-spore forming, Gram-positive rods forming the coryneform group which is heterogeneous. These bacteria were originally grouped in the genus *Corynebacterium* on the basis of morphological features. Indeed, coryneform bacteria have a particular mode of division which results in pairs of cells that have a V shape (diphtheroid arrangement). Phylogenetic analysis of 16S rRNA gene sequences allowed distinguishing *Clavibacter*, *Rathayibacter* and *Corynebacterium* species (Lee et al. 1997a). Currently, on the basis of chemotaxonomic and molecular traits, they are considered as different from *Corynebacterium* and as distinct genera which were reclassified in the three different families Micrococcaceae Microbacteriaceae and Nocardiaceae under the order *Micrococcales* while *Corynebacterium* belongs to the family Corynebacteriaceae, order Corynebacteriales. *Curtobacterium flaccumfaciens* comprises pathovars responsible of bacterial wilt, canker and leaf spot and silvering disease in diverse plants including bean, sugar beet, red beet, tulip bulb and common poinsettia (Collins and Jones 1983; Chen et al. 2007). Other pathogens belonging to the same family are *Leifsonia xyli* subsp. *xyli* (causal agent of ratoon stunting of sugarcane) and species of the widespread phytopathogen *Clavibacter* (Davis et al. 1984). Genus *Clavibacter* consists of only one species of quarantine significance in Europe, Canada and the United States, *Clavibacter michiganensis* which encompass plant pathogens residing mainly in the xylem vessels (as biotrophic phytopathogen) of economically important plants. *Rhodococcus fascians* is the causal agent of leaf and crown gall of various plants (Vereecke et al. 2003). Chemotaxonomy allow to distinguish all the *coryneforms*. *Arthrobacter* is the only coryneform that possess a cell wall type A₃a containing lysin (Collins et al. 1981). *Rhodococcus* has an A₁γ diaminopimelic acid (meso-A2 pm) in the cell wall and is the only coryneform that possess mycolic acids. The other *coryneforms* belonging to the family Microbacteriaceae have a B₂ type peptidoglycan (Davis et al. 1984; Park et al. 1993) which is considered as a rare peptidoglycan in the Actinobacteria. Within this family, while *Curtobacterium* possess a B₂β cell wall type containing the diamino acid D-ornithine, the other three genera possess a B2γ peptidoglycan

containing diaminobutyrate (DL-DAB in *Leifsonia* and *Clavibacter* and L-DAB in *Rathayibacter*). *Leifsonia* species could be distinguished from *Clavibacter* by their motility, and the presence of unsaturated menaquinones of 11 isoprene units (MK-11 menaquinones) as major quinone while *Clavibacter* are non motile and possess unsaturated menaquinones of 9 isoprene units (MK-9) (Park et al. 1993).

C—Order Streptomycetales encompasses few phytopathogenic species of only one genus *Streptomyces*.

In the following paragraphs, the main focus will be on the diversity of pathogens belonging to two most common pathogens: *Clavibacter michiganensis* and *Streptomyces* species.

The Genus *Clavibacter* Genus *Clavibacter* belongs to the family Microbacteriaceae (Park et al. 1993). The species *C. michiganensis* strains are pleomorphic rods, mesophile, oxidase-negative obligate aerobes, nitrate and nitrite reduction are negative (Davis et al. 1984), cell wall of DL diaminobutyrate type ($\beta_2\gamma$ type peptidoglycan), which contains also rhamnose, galactose and mannose, unsaturated menaquinones (MK-9) (Collins and Jones 1980; Park et al. 1993), mycolic acids are not present. Fatty acids are straight saturated chains, anteiso-branched (*ai*-17:0 and *ai*-15:0) (Collins and Jones 1980) and saturated iso-methyl branched acids. Principal phospholipids are phosphatidylglycerol and diphosphatidylglycerol and G+C content of the DNA is about 69–78 mol%. The genus *Clavibacter* includes five subspecies according to their host specificity (Davis et al. 1984; Eichenlaub et al. 2006) subsp. *michiganensis* (causal agent of bacterial canker of tomato and pepper), subsp. *insidiosus* (causal agent of Bacterial wilt of lucerne), subsp. *nebraskensi* (causal agent of bacterial wilt of Maize), subsp. *sepedonicus* (causal agent of bacterial ring rot of potato) and subsp. *tessellarius* (causal agent of leaf spot of wheat).

Various methods were used to detect *C. michiganensis* in infected materials. They include cultural methods (Carlson and Vidaver 1982; Franken et al. 1993), Biolog (Harris-Baldwin and Gudmestad 1996; Kaneshiro et al. 2006), bioluminescence imaging (Xu et al. 2011), immunofluorescence microscopy (Franken et al. 1993), enzyme-linked immunosorbent assay (ELISA) (Slack et al. 1996; Kaneshiro et al. 2006) and genotypic methods. For example, Kaneshiro et al. (2006) used Biolog system to profile biochemical characteristics including sole carbon source utilization to differentiate *C. michiganensis* subsp. *michiganensis* strains from seed borne saprophytes.

Although the five subspecies of *C. michiganensis* form a homogenous group according to their cell wall composition, they are phylogenically distant. In addition to their host specificity, they present few phenotypic differences such as colony morphology, pigmentation, bacteriocin production (Gross and Vidaver 1979), fatty acid composition (Henningson and Gudmestad 1991), cellular proteins (Carlson and Vidaver 1982) and physiology (Dye and Kemp 1977). However, they show nearly identical protein profiles (Carlson and Vidaver 1982) and other metabolic features (Louws et al. 1998) making difficult their differentiation on the basis of phenotypic traits. Nevertheless, their grouping based on phenotypic features was found to be consistent with their grouping on the basis of DNA-DNA relatedness (Döpfer et al. 1982).

Molecular methods were used to detect *C. michiganensis* subspecies and to assess diversity among isolates within a subspecies (Louws et al. 1998; de Leon et al. 2009). Recently, Q-PCR (real-time PCR) was developed and allowed to detect *C. michiganensis* subsp. *michiganensis* in pure cultures with a detection threshold of 10^3 cfu ml⁻¹ (Zhao et al. 2007; Luo et al. 2008). Also, attempts aiming to differentiate *Clavibacter* subspecies as well as to differentiate between saprophytes, non virulent and virulent *Clavibacter* strains have been performed by many researchers (Dreier et al. 1995; Dreier et al. 1997; Louws et al. 1998; Pstrik and Rainey 1999; Smith et al. 2001; Bach et al. 2003; Waleron et al. 2011; Jacques et al. 2012; Zaluga et al. 2013). Molecular methods include qualitative gene PCR (Dreier et al. 1995; Li and de Boer 1995; Slack et al. 1996; Waleron et al. 2011; Jacques et al. 2012) rep-PCR (Louws et al. 1998; Smith et al. 2001), Nested PCR (Lee et al. 1997b), RAPD-PCR (de Leon et al. 2009), SSR-PCR (Baysal et al. 2010), RT-PCR (Bach et al. 2003), rDNA sequencing (Kaneshiro et al. 2006) and DNA hybridization (Slack et al. 1996). For example, molecular studies allowed to establish that the subspeciation event between *C. michiganensis* subsp. *michiganensis* and subsp. *sepedonicus* have occurred 53,000 to 1,120,000 years ago (Bentley et al. 2008).

Analyses of nearly complete sequences (97–99%) of the 16S rRNA and sequences of the 16S–23S rRNA intergenic spacer region have shown high homogeneity between four *C. michiganensis* subspecies (Li and de Boer 1995) (1% dissimilarity in their 16S rRNA genes and 5% for the intergenic spacer region) suggesting the high conservative nature of the 16S rRNA genes and the intergenic spacer in this species. PCR-RFLP analysis of 16S rDNA succeeded to differentiate the five subspecies (Lee et al. 1997a). However, both PCR-RFLP of 16S rDNA and of 16S–23S ITS (ITS PCR-RFLP) failed to differentiate strains within subspecies (Lee et al. 1997a; de Leon et al. 2009). Rep-PCR was evaluated as a tool to differentiate the subspecies of *C. michiganensis* and showed a high discriminatory power. Conserved repetitive element within these subspecies could be the basis of genomic fingerprints generated by the DNA primers REP, ERIC and BOX, and gel electrophoresis. DNA fragments generated by rep-PCR support the current division of *C. michiganensis* into the five known subspecies (Louws et al. 1998). Similarly, RAPD-PCR as well as Real-time ITS-PCR (Q-PCR with specific fluorescent probes) of 16S–23S rRNA intergenic spacer genes also allowed differentiating these five sub species (Pstrik and Rainey 1999; Bach et al. 2003). Moreover, by using rep-PCR (BOX-PCR) and RAPD-PCR of intergenic spacer region techniques, it was possible to investigate genetic diversity beyond the level of subspecies and to evaluate the diversity among strains within *C. michiganensis* subsp. *michiganensis* (Pstrik and Rainey 1999; de Leon et al. 2009). BOX-PCR fingerprints revealed that *C. michiganensis* subsp. *michiganensis* showed the highest polymorphism within *C. michiganensis* subspecies (Smith et al. 2001) and allowed to distinguish four different fingerprint patterns A, B, C and D within this phytopathogen (Louws et al. 1998). In addition to *C. michiganensis* subsp. *michiganensis*, RAPD also allowed to differentiate between strains within the other subspecies *C. michiganensis* subsp. *sepedonicus* and *C. michiganensis* subsp. *insidiosus* (Pstrik and Rainey 1999).

However, all these methods including rep-PCR technique were not able to separate virulent and avirulent *C. michiganensis* subsp. *michiganensis* strains as they

had identical rep-PCR fingerprints (Louws et al. 1998). Recently, phylogenetic analyses based on RFLP fingerprints of housekeeping genes fragments of *recA*, *rpoB*, and *rpoD* allowed differentiating between the *C. michiganensis* subsp. and also differentiation of *C. michiganensis* subsp. *michiganensis* strains (Waleron et al. 2011). More recent work by Jacques et al. (2012) used polyphasic approach to study diversity among a worldwide collection of pathogenic and non pathogenic *C. michiganensis* subsp. *michiganensis*. This polyphasic approach integrated multi-locus sequence-based analysis and typing (MLSA and MLST) based on six housekeeping genes (*atpD*, *dnaK*, *gyrB*, *ppK*, *recA* and *rpoB*), genotypic analysis of 16S rRNA gene sequences and four pathogenicity determinants (*CelA*, *ChpC*, *Pat-1* and *PpaA*) combined to phenotypic features. Result shows that pathogenic *C. michiganensis* subsp. *michiganensis* are phylogenetically distinct from nonpathogenic *C. michiganensis* neighbor strains. This separation between pathogenic and non pathogenic *C. michiganensis* species was also confirmed by Zaluga et al. (2013) by using total DNA–DNA hybridization and sequence analysis of *gyrB* and *dnaA* genes. In another hand, it is well established that *C. michiganensis* subsp. *michiganensis* pathogenicity determinants are plasmid borne and loss of these virulence plasmids converts these biotrophic pathogens into simple endophytes (Eichenlaub and Gartemann 2011). These circular plasmids carrying pathogenicity genes are pCM1 (27.4 kbp) and pCM2 (70.0 kbp) (Gartemann et al. 2008). *CelA* gene encoding an endo- β -1, 4-glucanase and a plasmidic serine proteases encoding *pat-1* region (on plasmid pCM2), were reported as involved in virulence (Meletzus et al. 1993; Jahr et al. 2000). Moreover, a reduced virulence was noted after deletion *pat-1* region whereas cloning of this sequence to endophytic, plasmid-free isolates rendered them virulent forms (Dreier et al. 1997). Southern blot analysis of DNA repetitive sequence of *pat-1rep* showed that this region is common in virulent *C. michiganensis* subsp. *michiganensis* strains. In these experiments, it was detected only in virulent strains that carry DNA homologous to *pat-1rep*, whereas avirulent isolates lacked such homologies (Dreier et al. 1997).

Genus Streptomyces Belongs to the family Streptomycetaceae under the order Streptomycetales. In contrast to coryneform phytopathogens, this group is homogeneous, and contains species with the Chemotype I (L-Diaminopimelic acid). Pathogenic species include those causing scab diseases in potato and other plants such as *S. scabies*, *S. reticuliscabies*, *S. acidiscabies* and *S. ipome*. Phenotypically, they possess monopodial branched aerial hyphae of 0.5–2.0 μm (Loria et al. 2003) and the cell wall chemotype I: LL-DAP (LL-A₂pm) and *glycine* and sugars are absent.

Streptomyces scabies or *scabiei* (Lambert and Loria 1989b), *S. acidiscabies* (Lambert and Loria 1989a) *S. turgidiscabies* (Miyajima et al. 1998) are the most studied causal agents of the common scab of potato (*Solanum tuberosum*). Another pathogen *S. caviscabies* also causes the common scab (Goyer et al. 1996). *S. reticuliscabies* is the causal agent of netted scab of potato (Bouchek-Mechiche et al. 2000). Russet scab of potato is caused by *S. aureofaciens*. *Streptomyces ipomea*, is the causal agents of fibrous sweet potato (*Ipomea batata*) soil rot (Person and Martin 1940) a fibrous necrotic lesion that affect potato and consequently causes yield loss (Loria et al. 1997).

Common scab affects also a number of root crops including carrot, radish, beet and turnip (Bouchek-Mechiche et al. 2000). Variations in disease incidence and severity of common scab caused by *S. scabies* were often observed and attributed to regional differences (Wanner 2006; Haynes et al. 2006) and variation in the environment conditions (Lapwood et al. 1973). Symptoms generated by these pathogens are raised or pitted lesions, often described as corky lesions on tubers. In addition to a reduced potatoes quality and marketability, common scab disease is characterized by earthy odour of geosmin, secreted by actinomycetes, which can make the potatoes inedible. Scab disease causing Actinobacteria are well known for their production of characteristic phytotoxins, thaxtomins which are pathogenicity determinants (Wanner 2009). These phytotoxins are involved in aggressivity of pathogenic strain by inhibition of primary cell wall of growing tissues (Leiner et al. 1996; Loria et al. 2003). These phytotoxins induce inhibition of cellulose biosynthesis (Loria et al. 1997) and the release of cello-oligosaccharides from plant tissue which are considered as pathogenicity inducers (Johnson et al. 2007). The production of another phytotoxin concanamycin was also reported in *S. scabies* (Natsume et al. 1996). Scab disease results in considerable economic loss in all areas where potato crops are affected. While *S. scabies* is a worldwide pathogen, prevalent in most potato growing areas, limited geographic distribution is observed for *S. acidiscabies* and *S. turgidiscabies*. *S. acidiscabies* was found in northeastern United States (Lambert and Loria 1989a), Canada (St-Onge et al. 2008), Hokkaido in Japan (Tóth 2001), Korea (Park et al. 2003), *S. turgidiscabies* found in eastern Hokkaido, Japan (Miyajima et al. 1998), Finland (Kreuze et al. 1999), and Sweden (Lehtonen et al. 2004).

S. reticuliscabies, causal agents of netted scab of potato (Bouchek-Mechiche et al. 2000) has only been reported in European countries (Scholte and Labruyère 1985). Symptoms of netted scab lesions on potato tubers are superficial, brown lesions that appear as thickened, necrotic periderm that fractures in a netted pattern. Root necrosis, associated with netted scab, can result in yield losses (Loria et al. 1997). Symptoms of russet scab of potato are superficial, brown lesions on the skin of the tubers that are limited to the periderm. Necrosis does not affect roots and consequently, the disease does not affect potato yields but only the marketability of the tubers. The disease has been reported in North America as caused by *S. aureofaciens* (Faucher et al. 1993) and by *S. chelonium* in Japan (Natsume et al. 2005). The symptoms of netted and russet scab on the tuber surface are very similar as they are superficial and are never raised or pitted. In addition, both russet and netted scabs increase in severity in response to high soil moisture. However, they belong to distinct species (Oniki et al. 1986; Faucher et al. 1993). They are different in the optimal conditions for disease development (Bouchek-Mechiche et al. 2000). Indeed, the optimum temperature for russet scab is higher than for netted scab. In addition, as mentioned above, while russet scab lesions are not associated with root necrosis (Loria et al. 1997), netted scab is associated with necrosis of all underground parts of the potato plant, including roots (Scholte and Labruyère 1985).

Many research works focused on diversity among scab disease inducing pathogens (Doering-Saad et al. 1992; Bramwell et al. 1998). Studies indicated that differ-

ent species are able to coexist in the same field and even the same tuber (Leiminger et al. 2013). Based on morphological, chemical and genomic characteristics, it was suggested to assign *S. scabiei* to the *Diastatochromogenes* cluster which encompasses *S. diastatochromogenes* and other *Streptomyces* species (Lambert and Loria 1989b; Healy and Lambert 1991). Infact, it was observed that strains of *S. scabies* had 16S rRNA gene sequences similar to those of *S. diastatochromogenes* (Takeuchi et al. 1996). Phenotypically, *S. scabies* group encompass species with grey spores borne in spiral chains, able to utilize the nine ISP (International Streptomyces project) sugars (Shirling and Gottlieb 1966), to produce melanine (Lambert and Loria 1989b) and sensitive to streptomycin (Bouchek-Mechiche et al. 1998). It was advocated that production of melanoid pigments is associated with pathogenicity in *S. scabiei* (Lambert and Loria 1989b). However, the discovery of pathogenic strains with no production of such pigments (Takeuchi et al. 1996; Dees et al. 2012) and their production by non-pathogens ascertain that their production is not essential for the pathogenicity. Lambert and Loria (1989a, b) used phenotypic traits to characterize *streptomyces* that cause common potato scab disease responsible of potato common scab disease (Locci 1994). In this study, virulent pathogens were classified into two species: *Streptomyces scabiei* and *Streptomyces acidiscabiei* (pathogenic form in soils with a pH below 5.2).

However, genetic diversity of the pathogen is a factor of great importance (Wanner 2006). Molecular studies have shown a great diversity among strains of this species (Healy and Lambert 1991; Paradis et al. 1994). DNA relatedness support genetic diversity among strains classified phenotypically as *S. scabies*. Healy and Lambert (1991) showed that the level of DNA relatedness between two strains phenotypically characterized as *S. scabies* could be as low as 20%. The levels of DNA relatedness among *S. scabies* resulted in a diversity that should make them belong to more than one species (Healy and Lambert 1991; Kers et al. 2005) implying that phenotype is not sufficient to assess phylogenetic relatedness between *S. scabies* species. Similar results were found by Paradis et al. (1994) who used PAGE and DNA–DNA hybridizations to elucidate the taxonomy of pathogenic *S. scabies* isolates from eastern Canada. In this study, results of protein profiling on SDS-PAGE correlated well with DNA–DNA hybridization and showed that *S. scabies* isolates were divided into two genetic clusters. However, similarly to phenotypic characterization (Lambert and Loria 1989a) and fatty acid analyses (Paradis et al. 1994), DNA–DNA hybridization cannot distinguish saprophytic isolates from pathogenic *S. scabies* species (Healy and Lambert 1991; Paradis et al. 1994).

Bouchek-Mechiche et al. (1998) isolated several *S. scabiei* strains from common scab and netted scab on potatoes and other root vegetables in France and other countries. They showed that *S. scabies* phenon form a relatively phenotypically heterogeneous group. Thereafter, authors described three subphenons and suggested that *S. scabiei* phenetic cluster could be separated into three genomic species including the subphenon *S. scabiei* and probably two new species. Furthermore, genomic relatedness evaluated by DNA–DNA hybridization allow to specify relatedness between all those distantly related genomogroups (sharing low DNA relatedness) and confirmed that two of the three phenotypic groups associated with

common scab correspond to genomospecies (within which homology is equal to or greater than 70%), represented by new species; *S. europaeiscabiei* and *S. stelliscabiei* (Bouchek-Mechiche et al. 2000). The phenotypic study also allowed describing another phenon, different from *S. scabiei* and *S. acidiscabiei* phenotypic groups, which encompass netted scab associated strains (Bouchek-Mechiche et al. 1998). For this phenon, DNA–DNA hybridization allowed giving further information leading to describe in this phenotypic group, another new species, *S. reticuliscabiei* (Bouchek-Mechiche et al. 2000). Moreover, in this study, DNA–DNA hybridization succeeded to differentiate between pathogenic and saprophytic strains of *S. scabies* as all the common scab pathogenic strains were grouped into three genomospecies strictly correlated with the phenotypic clusters, while saprophytic *Streptomyces* sp. strains form a separate group. After that, *S. europaeiscabiei* was also reported in US and Canada (Bukhalid et al. 2002; Wanner 2006). While molecular studies allowed divisions within the *S. scabies* group into distinct species despite its phenotypic uniformity, recent investigations by DNA–DNA hybridization and phylogenetic comparisons of 16S rRNA gene sequences demonstrated that *S. turgidiscabies* and *S. reticuliscabiei* strains are too close (high DNA–DNA relatedness of 75–88%) and should constitute a single species although they are causal agents of two different diseases (Bouchek-Mechiche et al. 2006). It could be advocated that subspecies could be delineated for these two genetically closely related species. However, to avoid any confusion as those names are largely used by phytopathologists, these authors proposed, for the purpose of practicability that the existing distinct species names could be retained. Recent works described other new potato scab disease causing agents, *S. luridiscabiei*, *S. puniscabiei* and *S. niveiscabiei* in Korea (Park et al. 2003). Since it was discovered in France by Bouchek-Mechiche et al. (1998), *S. europaeiscabiei* was reported in other areas in Europe (Dees et al. 2012; Leiminger et al. 2013) and USA (Wanner 2009) suggesting that this species may be as worldwide distributed as *S. scabiei*. Aiming to investigate internal transcribed spacer (ITS) region could be used as alternative to 16S rRNA sequences. Song et al. (2004b) used ITS sequences to study *Streptomyces* strains isolated from potato scab tuber lesions. Except for *S. scabiei* and *S. europaeiscabiei* which were differentiated, authors confirmed that ITS regions are not useful in phylogenetic analysis of scab inducing pathogens *Streptomyces* species.

On the other hand, pathogenicity in *Scabiei* is most likely to be related to virulent genes and several studies have been performed to characterize genomes of factors involved in the pathogenicity of scab inducers (pathogenicity factors). Among these factors, the pathogenic determinant thaxtomins genes (*txtAB*, *txtC*, and *nos*) are the most studied. Non pathogenic *S. scabies*, *S. acidiscabies* and *S. turgidiscabies* species are presumed to be lacking of thaxtomins and their occurrence has been reported by several reports (Faucher et al. 1992; Wanner 2006, 2009). Thaxtomins are considered as pathogenicity determinants. Dees et al. (2012) showed that the *txtAB* sequences of different species including *S. scabiei* and *S. turgidiscabiei* are divergent. However, the presence of this gene is well correlated with the pathogenicity (Wanner 2009). Thus, *TxtAB* operon amplification could be used as a diagnostic tool for the detection and quantification of common scab pathogenic *Streptomyces*

in potato tubers and soil samples (Flores-González et al. 2008; Qu et al. 2008). In addition to thaxtomins, other factors involved in the pathogenicity also include *Nec1*; a necrosis-inducing protein which is required for colonization of plant roots (Joshi et al. 2007) and cytokinin (Joshi and Loria 2007) both found in *S. turgidiscabies* and tomatinase enzyme (Seipke and Loria 2008), a pyochelin siderophore (Seipke et al. 2011) and a coronafacic acid-like (Bignell et al. 2010) found in *S. scabies*. In *S. turgidiscabies*, Kers et al. (2005) characterized a mobile pathogenicity island (PAI) which is a large single 425-kb fragment that contains three loci which are genes for synthesis of the pathogenicity determinant thaxtomin (*txtAB*, *txtC*, and *nos*), genes for tomatinase (*tomA*) synthesis and *nec1* gene. It appears that pathogenicity is an acquired phenotype which resides on such a virulence factors as PAI genome confers the ability to cause disease when transferred to a saprophytic streptomycete (Kers et al. 2005). In addition, Bukhalid et al. (1998), demonstrated that the virulence gene, *nec1*, have been horizontally transferred between strains of *S. scabiei*, *S. acidiscabies* and *S. turgidiscabies*. In another work, Healy et al. (1999), by analyzing diversity in the RFLP profiles of an insertion sequence (IS) element, demonstrated that the direction of the horizontal transfer has been from *S. scabiei* to *S. acidiscabies* and *S. turgidiscabies*. It had also been hypothesized that horizontal transfer occurred from an *S. scabies* strain into saprophytic species to create *S. acidiscabies* and *S. turgidiscabies* (Healy et al. 1999; Bukhalid et al. 2002). However, Wanner (2006) isolated some strains holding genes for biosynthesis of thaxtomin which are pathogenic although they lack one or more genes characteristic of the PAI (Wanner 2006). Also, necrogenic factor *nec1* was found in *S. acidiscabies* and *S. turgidiscabies* whereas *S. ipomoea* strains do not carry it (Bukhalid et al. 2002). Actually, *nec1* is not required for pathogenicity or thaxtomin A production, as isolates holding *txtAB* and lacking the *nec1* gene are naturally occurring (Wanner 2006; Flores-González et al. 2008). Moreover, it was also reported that one *txtAB*⁺ *Streptomyces* sp. strain lacking in *nec1* was more virulent than *S. scabies* (Wanner 2007a, b). All these observations suggest that that thaxtomin and *nec1* genes are independent features and that this feature should be taken into account in studies of the diversity of pathogenic and non pathogenic strains.

3.4 Conclusion

In this chapter, phenetic and genotypic diversity of most important Actinobacteria were reviewed. Taxonomy provides the foundation for studies on bacterial diversity in general. Taxonomically, Actinobacteria represent one of the most diverse groups of bacteria. Currently, valid publication of new names and new nomenclatural combinations can only be made by publication in the International Journal of Systematic and Evolutionary Microbiology (IJSEM) and published in the Approved List of Bacterial Names (Skerman et al. 1980, 1989). The taxonomy of the Actinobacteria is continuously evolving and periodically improved. Numerous strains were reclassified as new genera or reclassified in other Families. The example of *Coryneforms*

of the family Microbacteriaceae which were previously classified as belonging to the genus *Corynebacterium* is well known (Collins and Jones 1983; Goodfellow 1984; Zgurskaya et al. 1993; Evtushenko et al. 2000a). Also, Actinobacteria previously designated as species of *Clavibacter* are now reclassified as new genera (Sasaki et al. 1998; Evtushenko et al. 2000a). Within the same family, another example is the recent reclassification of *Leifsonia ginsengi* as *Herbiconiux ginsengi* as new chemotaxonomic and phylogenetic data support differentiation from recognized species of the genus *Leifsonia* (Behrendt et al. 2011). Another example is that of *Streptovorticillium* which is currently considered as a synonym of *Streptomyces* as they were found to be phylogenetically closely related on the basis of 16S and 23S rRNA analysis (Witt and Stackebrandt 1990). In the present chapter, new arrangements of the Bergey's Manual of Systematic Bacteriology (Volume 5) (Ludwig et al. 2012) where the phylum Actinobacteria is subdivided into six classes are adopted. Among them, the class Actinobacteria comprises 16 orders and 43 families. Actinobacteria play either beneficial or adverse effects in crop systems. Increased interest in low-input agriculture had encouraged the search for microbial inoculants of Actinobacteria showing beneficial effects to plants i.e., disease suppression, plant fitness enhancement and abiotic stress tolerance. Thus, their diversity in relation to plant life is one of the major practical aspects related to crop health and improvement. They were isolated as phyllosphere, rhizosphere, rhizoplane or endosphere inhabitants. In another hand, numerous studies established that their diversity is usually influenced by plant species and responses to environmental changes that underlie shifts in their communities.

On the other hand, enhancing our knowledge on the diversity of rhizospheric, endophytic and pathogenic Actinobacteria could be of great importance for sustainable agriculture and plant diversity preservation. Both culture dependant and independent methods are used to assess Actinobacterial diversity. Most studies combined these methods. Unfortunately, culture dependant methods had the disadvantage of being limited as fewer than 1 % of bacterial species are cultivable at present (Giovannoni and Stingl 2005). With the development of new culture independent methods especially 16S rDNA phylogenetic analyze members of uncultured organisms are revealed (Heuer et al. 1997). Therefore, less biased molecular-based approaches provided extended data for Actinobacterial diversity associated with plant-soil systems. Informations on their diversity could be improved by improvement in isolation methods. Also, unexplored ecological niche or underexploited habitats such as mountainous ecosystems (Dolotkeldieva and Totubaeva 2006) and the rhizosphere and endosphere of not yet studied plants could be pursued as sources of new Actinobacteria. Particularly, indigenous plants might be a rich source of novel actinomycetes species (Bouizgarne et al. 2009; Kirby and Meyers 2010). Recently, a growing number of Actinobacteria has been discovered from not yet explored Asian and South African plants or Australian native trees (Qin et al. 2009a; Kirby and Meyers 2010; Kaewkla and Franco 2010a, b, c). However, 16S rRNA analyses and other molecular techniques based on PCR suffer from insufficient resolution as PCR biases can lead to biases in molecular-based diversity studies (Kirk et al. 2004). For instance, von Wintzingerode et al. (1997) reported that sequences with

high C+G microorganisms (including Actinobacteria) are less preferentially amplified and separate less efficiently in the denaturing step of PCR than bacteria with lower G+C content. Continued effort to improve cultivation techniques, along with the application of culture-independent methods, within a polyphasic framework will help reveal the role of Actinobacterial diversity in soils. In addition, current taxonomy is based on phenotypic and the analysis of the conserved sequence of the 16S rDNA. The improvement of DNA extraction from soil samples with high purity is of great importance for sensitivity and reproducibility of results. Such improvement is enabled by Soil DNA Isolation kits (MoBio Laboratories Inc., Solana Beach, CA) (Gonzalez-Franco et al. 2009). Also, improvement of the use of specific primers and environmental clone libraries (McCaig et al. 1999; Rintala et al. 2001), DNA hybridization (Zaluga et al. 2013), whole-genome microarray analysis (Dees et al. 2012) and the 16S rRNA gene Microarrays PhyloChip technology (Weinert et al. 2011) which proved to reveal more bacterial diversity in environmental samples than clone library (DeSantis et al. 2007) will constitute a powerful approach for studying the Actinobacterial diversity. Still, a great development of the diversity knowledge could be the result of the great ongoing progress in their taxonomy. For this purpose, generation of trees based on whole genome sequences (Normand et al. 2007; Ventura et al. 2007) and use of housekeeping genes and Multilocus sequence typing (MLST) that brought a new dimension into the elucidation of genomic relatedness at the inter- and intraspecies level (Dahllöf et al. 2000) are of great interest. This will allow to further elucidating the diversity and interrelations within Actinobacteria and between Actinobacteria and other closely or distantly related microorganisms where they reside and cohabit i.e., bulk soil, rhizosphere, or endosphere as well as to provide more insight in interactions between Actinobacteria and plants.

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Chapter 4

Root-Nodule Bacteria of Legumes Growing in Semi-Arid African Soils and Other Areas of the World

Flora Pule-Meulenberg

Abstract In Africa, arid and semi-arid soils are characterized by a diverse occurrence of plants including nodulated legumes which are adapted to the prevailing and often harsh environmental conditions. Such soils also harbour a large diversity of indigenous rhizobial symbionts (comparable to legume diversity) and other bacterial endophytes that have a potential to be developed into elite strains to be used as inoculum. However, very few studies have investigated the diversity of root-nodule bacteria on African soils, the main reason being that funds for research are often lacking. Discoveries of new rhizobial species are to be expected from different soil ecologies that are likely to be inhabited by various types of microorganisms. It is concluded that the semi-arid and arid African soils have rhizobia that is possibly as equally diverse as their host legumes. Research in these areas is likely to find novel species with a great potential for use in agricultural systems.

4.1 Introduction

Arid and semi-arid soils are first and foremost characterized by shortage or lack of available water. In Africa, in addition to low moisture content, such soils are known to have low nitrogen and phosphorus, low organic matter, and low amounts of exchangeable cations. Their formation is conditioned by their lack of moisture or capacity to retain it, extreme temperature, and wind and soil microorganisms. Therefore, plants that are able to survive have adapted to their aridity and low fertility status. Of special importance are nodulated legumes, which are highly diverse and are found in three main tropical vegetation types including tropical rain forests, dry forests and woody savannas (LPWG 2013).

The rhizosphere is a highly important source of organic material in soils. It contributes to soil organic matter directly through the release of soil root material and its decomposition and indirectly through root exudation, which stimulates microbial activity and biomass (Lynch 2010).

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Leguminous plants (Leguminosae) and the non-legume *Parasponia* (Ulmaceae) have a symbiotic relationship with five genera of bacteria in the family Rhizobiaceae that have the ability to infect roots and form N_2 -fixing nodules. The nodules usually develop on plant roots but may be formed on the stem. As the nodules are readily identifiable, rhizobia have been studied and classified since the beginning of scientific research in the field of bacteriology (Bartsev et al. 2003). The five genera of root-nodule bacteria (collectively called “rhizobia”), include *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Azorhizobium* and *Ensifer* (formerly *Sinorhizobium*) (Sprent 2001), all belong to α -proteobacteria. Rhizobia are genetically diverse and physiologically heterogeneous group of bacteria that were nevertheless classified together by their ability to nodulate various members of the family Leguminosae. Rhizobia normally occur in the rhizosphere, root surface, in the large number as a result of the nutrients secreted by the plant in the form of root exudates (Van Berkum and Eardly 1998). Rhizobia is an important group of microorganisms both ecologically and agriculturally (Kloepper et al. 1980; Young and Haukka 1996), as the establishment of beneficial bacteria on root system of plants via seed bacterization has long been of a major interest to agricultural researchers (Kloepper et al. 1989). In addition, a *Burkholderia* strain belonging to the β -proteobacteria was isolated from root nodules of *Aspalathus carnosa* in South Africa with nodulating ability on siratro (Moulin et al. 2001). *Ralstonia taiwanensis*, another β -proteobacteria was isolated from nodules of *Mimosa* species, and has been similarly confirmed to nodulate and fix N_2 in the homologous *Mimosa* host plants (Chen et al. 2003). *Oryza sativa* has also played host to endophytic strain which include β -proteobacteria *A. xylosoxidans* (Mano and Morisaki 2008).

The association between rhizobia and some members of the Leguminosae accounts for 80% of biologically fixed nitrogen and contributes 25 to 30% of the worldwide protein intake (Vance 1996). This is particularly important for the resource-poor African farmers who depend on nodulated legume parts such as cowpea for fresh young leaves used as spinach and fresh young pods and grain for their protein intake. The symbiotic relationship between the diazotrophic rhizobia and legumes (e.g., Soybean, Clover, Common bean) can provide large amount of nitrogen to the plant and has a significant impact on agriculture. Despite this, very few studies target the optimisation of the legume/rhizobia symbiosis in semi-arid Africa. Rhizobia in semiarid to arid soils is able to live in harsh soil conditions such as extreme temperature and low moisture and that they effectively nodulate plants growing in those soils is proof that they can survive.

The most well-known traditional use of rhizobia has been in agricultural systems where the bacteria colonizes root nodules and form a symbiotic relationship with a plant, whereby the plant serves as a carbon substrate for the micro-organism and the bacteria supplies nitrogen to the plant. This chapter discusses (i) a general taxonomic history of root nodule bacteria, (ii) methods for systematic studies of root-nodule bacteria, (iii) global diversity and taxonomy of root nodule bacteria and (iv) rhizobial symbionts from African soils.

4.2 Brief History of Root-Nodule Bacteria

The term “root nodule bacteria, often used to refer to the exclusive presence of rhizobia in nodules, should be redefined with root nodule bacteria capable of acting as a plant growth promoting bacteria/rhizobacteria with specialized ability to colonize the internal tissue of roots of the plant (Selvakumar et al. 2007; Palaniappan et al. 2010). Beijerinck (1888) was the first to culture the first root-nodule bacteria, which he named *Bacillus radicola* (Eckhardt et al. 1931). After many years of disputes concerning microorganisms associated with root nodules, *B. radicola* was placed in Bergey’s Manual of Determinative Bacteriology under the genus *Rhizobium*, with two species *R. radicola* and *R. leguminosarum* (Baldwin and Fred 1929). Soon after, *Rhizobium trifolii*, *R. phaseoli*, *R. meliloti*, *R. japonicum* and *R. lupini* (Fred et al. 1932) were also recognized as distinct species, based on their ability to nodulate certain legumes. This use of cross-inoculation groups was discontinued in 1944 due to unexplainable incongruous plant and bacteria reactions (Burton 1979). Growth characteristics, production of acid, serology, G+C contents, numerical taxonomy, DNA hybridization and phage susceptibility were later the focus of differentiation (Burton 1979; van Berkum and Eardly 1998) and the formal adoption of the new approved lists of bacteria lists only took place in 1980 (Skerman et al. 1980).

Long back, Smith and Townsend (1907) isolated crown-gall inducing bacteria, which they named *Bacterium tumefaciens*. Much later, Conn (1942) concluded that *Alcaligenes radiobacter*, a soil bacterium first isolated in 1902, was similar to crown gall-inducers and legume nodule bacteria based on morphological and physiological characteristics, and proposed a new genus, *Agrobacterium*. The approved list of bacterial names included *A. tumefaciens*, *A. radiobacter*, *A. rhizogens* and *A. rubi* (Skerman et al. 1980). Much later, *A. vitis* (Ophel and Kerr 1990) and *A. larrymoorei* (Bouzar and Jones 2001) were also described.

The past few years have seen a plethora of interesting developments in molecular microbiology for bacterial classification and identification. Of particular interest are studies of rhizobial diversity since they have a direct impact on sustainable agriculture. Van Berkum and Eardly (1998) described classification, nomenclature and identification as the three areas of systematics that are necessary for describing organisms. The international subcommittee on the taxonomy of rhizobium and agrobacterium proposed the following as minimal standards for the description of root- and stem-nodulating bacteria: symbiotic performance with selected hosts, cultural and morphological characteristics, DNA-DNA hybridization and 16S rDNA analysis, DNA restriction fragment length polymorphism and multilocus enzyme electrophoresis (Graham et al. 1991). This section will discuss some concepts on taxonomic techniques used to study bacteria. Systematic studies for studying bacteria can be either phenotypic or genotypic.

In phylogenetic analysis of 16S data, it is important to include both related and unrelated organisms in the analysis (Vandamme et al. 1996). It is better to target the conserved region as information will be lost when comparing only the hypervariable

regions, since a substantial amount of difference is not concentrated in these regions (Stackebrandt and Goebel 1994).

Phenotypic methods comprises all those that are non-DNA or RNA based. Bacterial traits such as morphological, physiological and biochemical features are used to formally describe the taxa, from species and subspecies up to genus and family level. Phenotypic bacterial identification methods have numerous strengths but often fail because the phenotype may be variable and subject to biases of interpretation. Therefore, to reliably describes and differentiate taxa, often genotypic methods are required (Vandamme et al. 1996).

To form an effective symbiosis, rhizobia require several classes of specific genes; these include *nod* genes which encode the production of nod factors, which stimulate the plants to produce symbiotic nodules. The *nod* genes are found in all rhizobia and code for nod factor which are responsible for nodule formation (Lindström et al. 1995).

A lot of methods based on DNA amplification by polymerase chain reaction (PCR) for typing bacteria have come up in the past 20 years. Ideally, the entire genome of an isolate would provide definite typing. Although technically feasible, it is very expensive and therefore, more practical DNA analysis methods, termed DNA fingerprinting techniques have been developed. These include techniques such as random amplified polymorphism DNA (RAPD) assay (Williams et al. 1990), rep-PCR and restriction fragment length polymorphism (RFLP). In RAPD, short random sequence primers are used to initiate amplification of random regions on the bacterial genome, while the rep-PCR technique relies on amplifying repetitive DNA sequences separating longer, single copy sequences to produce amplicons of varying lengths that can be separated by electrophoresis. In RFLP analysis, the amplified DNA sample is digested by restriction enzymes and the resulting restriction fragments separated according to their lengths by gel electrophoresis. Pulse-field gel electrophoresis (PFGE) allows the electrophoretic separation of low number of large DNA restriction fragments. This is the method of choice in the typing of human bacterial pathogens and investigations of disease outbreaks, but is a relatively costly method. All of these DNA analyses are termed random whole-genome analysis techniques and they rely on the presence of repetitive elements which they all target.

Outside random whole-genome techniques, another group of techniques is called specific gene variation. Approaches for these set of techniques can be either single-locus or multi-locus. The single locus approach uses highly variable genes that are implicated in causing disease (Gürtler and Mayall 2001). In contrast, multilocus approaches comprise of multilocus sequence typing (MLST) as well as analysis of multigene families such as *rrn* operons and tRNA genes (which include 16S rRNA variations, 16S–23S rDNA IGS region typing and others). The 16S–23S IGS region exhibits a large degree of sequence length and frequency variation even among closely related species (Massol-Deya et al. 1995), and as such, members of α -Proteobacteria whose length vary between 800 to 1200 nucleotides are suitable (Normand et al. 1996). Gürtler and Mayall (1999) showed that while some parts of IGS were highly variable, it also had some highly conserved blocks. Because of the

variation, analysis of this region results in a high level of discrimination. Laguerre et al. (1996) were able to show differences between biovars of *R. leguminosarum* using IGS.

In the last decade, as a result of the widespread use of PCR and DNA sequencing, 16S rRNA sequencing has played a pivotal role in the accurate identification (Laguerre et al. 1997; Gürtler 1999; Moschetti et al. 2004) and phylogeny (Woese 1987) of bacterial isolates. One of the reasons why the genes for the 16S have become indispensable in classifying bacteria, particularly rhizobia, is that there are many published sequences, so that new ones can be placed in context (Young and Haukka 1996). For bacterial identification, 16S rDNA sequencing is particularly important in the case of bacteria with unusual phenotypic profiles and slow-growing bacteria (Petti et al. 2005). Identification of root nodulating bacteria based on recent molecular approaches, greater than 70% DNA–DNA homology coupled to distinctive phenotypic characters was the main criteria for defining species. However, in a few cases strains sharing lower homology (40–60%) have also been found within a species, viz. *R. tropici* type A and B (Martinez-Romero et al. 1991) and *Mesorhizobium plurifarium* (De Lajudie et al. 1998).

So far, advances made in bacterial systematics can to a large extent be ascribed to the development of molecular techniques. Each of the available techniques has its own level of discrimination and thus should be applied to complement each other. However, the choice of technique will to a large extent be determined by the objectives of the study and often the cost implications.

4.3 Global Rhizobial Diversity and Taxonomy

Currently, 125 species in 13 genera are recognised as rhizobia (Lindström et al. 2010; Maheshwari et al. 2013), figures that are probably much lower than the actual number of rhizobial species, had the nodulation status of many legumes been known. It is possible that the actual number of rhizobial species may be similar to the number of legume species (Young and Haukka 1996). The known genera include *Azorhizobium*, *Bradyrhizobium*, *Rhizobium*, *Ensifer*, *Mesorhizobium*, *Methylobacterium*, *Burkholderia*, *Cupriavidus*, *Microvirga*, *Ochrobactrum*, *Phyllobacterium*, *Devosia*, *Shinella* and *Herbaspirillum*. Of the 125 species, two belongs to genus *Azorhizobium*, 16 to *Bradyrhizobium*, 15 to *Mesorhizobium*, 55 to *Rhizobium*, 15 to *Ensifer*, 1 to *Methylobacterium*, 3 to *Microvirga*, 2 to *Ochrobactrum*, one to *Phyllobacterium*, 11 to *Burkholderia*, two to *Cupriavidus*, 1 to *Shinella* and 1 to *Herbaspirillum* (Table 4.1).

4.3.1 *Azorhizobium*

For a long time, *A. caulinodans* (Dreyfus et al. 1988) was the only named species in this genus. This species nodulates both stems and roots of *Sesbania rostrata*.

Table 4.1 Global list of currently described root-nodule bacteria as at January 2014

Host plant/place of isolation	Genus	Species	Reference	Origin
<i>Sesbania rostrata</i>	<i>Azorhizobium</i>	<i>caulinodans</i>	Dreyfus et al. (1988)	Senegal
<i>Sesbania virgate</i>	<i>Azorhizobium</i>	<i>doeberienerae</i>	Moreira et al. (2006)	Brazil
<i>Glycine max</i>	<i>Bradyrhizobium</i>	<i>japonicum</i>	Jordan (1982)	USA
<i>Chamaecytisus</i> sp., <i>Lupinus</i> sp.	<i>Bradyrhizobium</i>	<i>canariense</i>	Vinuesa et al. (2005)	Canary Islands
<i>Cytisus villosus</i>	<i>Bradyrhizobium</i>	<i>cytisi</i>	Chahboune et al. (2012)	Morocco
<i>Glycine max</i>	<i>Bradyrhizobium</i>	<i>elkanii</i>	Kuykendall et al. (1992)	Brazil
<i>Beta vulgaris</i>	<i>Bradyrhizobium</i>	<i>betae</i>	Rivas et al. (2004)	Spain
<i>Aechynomene indica</i>	<i>Bradyrhizobium</i>	<i>denitrificans</i>	Van Berkum and Eardly (2002)	USA
<i>Lespedeza</i> sp., <i>Medicago</i> sp., <i>Melilotus</i> sp.	<i>Bradyrhizobium</i>	<i>yuanmingense</i>	Yao et al. (2002)	China
<i>Glycine max</i>	<i>Bradyrhizobium</i>	<i>huanghuai-haiense</i>	Zheng et al. (2012)	China
<i>Pachyrhizus erosus</i>	<i>Bradyrhizobium</i>	<i>jicamae</i>	Ramírez-Bahena et al. (2009)	Spain
<i>Entada koshunensis</i>	<i>Bradyrhizobium</i>	<i>iriomotense</i>	Islam et al. (2010)	Japan
	<i>Bradyrhizobium</i>	<i>lablabi</i>	Chang et al. (2011)	
<i>Glycine max</i>	<i>Bradyrhizobium</i>	<i>liaoningense</i>	Xu et al. (1995)	China
<i>Pachyrhizus erosus</i>	<i>Bradyrhizobium</i>	<i>pachyrhizi</i>	Ramírez-Bahena et al. (2009)	Spain
<i>Glycine max</i>	<i>Bradyrhizobium</i>	<i>daqingense</i> sp.	Wang et al. (2013a)	China
<i>Arachis hypogaea</i>	<i>Bradyrhizobium</i>	<i>arachidis</i>	Wang et al. (2013b)	China
<i>Cytisus villosus</i>	<i>Bradyrhizobium</i>	<i>rifense</i>	Chahboune et al. (2012)	Morocco
<i>Albizia kalkora</i>	<i>Mesorhizobium</i>	<i>albiziae</i>	Wang et al. (2007)	China
<i>Alhagi sparsifolia</i>	<i>Mesorhizobium</i>	<i>alhagi</i>	Chen et al. (2010)	China
<i>Amorpha fruticosa</i>	<i>Mesorhizobium</i>	<i>amorphae</i>	Wang et al. (1999)	China
<i>Biserrula pelecinus</i>	<i>Mesorhizobium</i>	<i>australicum</i>	Nandasena et al. (2009)	Australia
<i>Alhagi sparsifolia</i>	<i>Mesorhizobium</i>	<i>camelthorni</i>	Chen et al. (2011)	China
<i>Caragana bicolor</i>	<i>Mesorhizobium</i>	<i>caraganae</i>	Guan et al. (2008)	China
<i>Prosopis alba</i>	<i>Mesorhizobium</i>	<i>chacoense</i>	Velázquez et al. (2001)	Argentina
<i>Cicer arietinum</i>	<i>Mesorhizobium</i>	<i>ciceri</i>	Nour et al. (1994), Jarvis et al. (1997)	Spain
Wild legumes growing in desert soils of Xinjiang	<i>Mesorhizobium</i>	<i>gobiense</i>	Han et al. (2008)	China
<i>Astragalus sinicus</i>	<i>Mesorhizobium</i>	<i>huakuui</i>	Chen et al. (1991), Jarvis et al. (1997)	China
<i>Lotus</i> sp.	<i>Mesorhizobium</i>	<i>loti</i>	Jarvis et al. (1982, 1997)	New Zealand
<i>Cicer arietinum</i>	<i>Mesorhizobium</i>	<i>mediterraneum</i>	Nour et al. (1995), Jarvis et al. (1997)	Spain
<i>Anthyllis vulneraria</i>	<i>Mesorhizobium</i>	<i>metalliduran</i>	Vidal et al. (2009)	France
<i>Cicer arietinum</i>	<i>Mesorhizobium</i>	<i>muleiense</i>	Zhang et al. (2012)	China
<i>Biserrula pelecinus</i>	<i>Mesorhizobium</i>	<i>opportunum</i>	Nandasena et al. (2009)	Australia

Table 4.1 (continued)

Host plant/place of isolation	Genus	Species	Reference	Origin
<i>Acacia senegal</i> , <i>Prosopis juliflora</i> , <i>Acacia</i> sp., <i>Leucaena</i> sp., <i>Chamaecrista ensiformis</i>	<i>Mesorhizobium</i>	<i>plurifarum</i>	De Lajudie et al. (1998)	Senegal
<i>Robinia pseudoacacia</i>	<i>Mesorhizobium</i>	<i>robiniae</i>	Zhou et al. (2010)	China
<i>Astragalus adsurgens</i>	<i>Mesorhizobium</i>	<i>septentrionale</i>	Gao et al. (2004)	China
<i>Caragana</i> sp.	<i>Mesorhizobium</i>	<i>shangrilense</i>	Lu et al. (2009b)	China
<i>Astragalus</i> sp.	<i>Mesorhizobium</i>	<i>silamurunense</i>	Zhao et al. (2012)	China
Wild legumes growing in desert	<i>Mesorhizobium</i>	<i>tarimense</i>	Han et al. (2008)	China
<i>Astragalus adsurgens</i>	<i>Mesorhizobium</i>	<i>temperatum</i>	Gao et al. (2004)	China
Indian tropical leguminous plant	<i>Mesorhizobium</i>	<i>thiogangeticum</i>	Ghosh and Roy (2006)	India
<i>Caragana</i> , <i>Glycyrrhiza</i>	<i>Mesorhizobium</i>	<i>tianshanense</i>	Chen et al. (1995), Jarvis et al. (1997)	China
<i>Anagyris latifolia</i> and <i>Lotus berthelotii</i>	<i>Mesorhizobium</i>	<i>tamadayense</i>	Ramírez-Bahena et al. (2012)	Canary Islands
<i>Cicer arietinum</i>	<i>Mesorhizobium</i>	<i>muleiense</i>	Zhang et al. (2012b)	Xingjiang, China
Agroforestry legume trees	<i>Mesorhizobium</i>	<i>abyssinicae</i>	Degefu et al. (2012)	Ethiopia
Agroforestry legume trees	<i>Mesorhizobium</i>	<i>hawassense</i>	Degefu et al. (2012)	Ethiopia
Agroforestry legume trees	<i>Mesorhizobium</i>	<i>shonense</i>	Degefu et al. (2012)	Ethiopia
<i>Astragalus sinicus</i>	<i>Mesorhizobium</i>	<i>qingshengii</i>	Zheng et al. (2012)	South East China
<i>Astragalus luteolus</i> , <i>Astragalus ernestii</i>	<i>Mesorhizobium</i>	<i>sangaii</i>	Zhou et al. (2013)	China
Hexachlorocyclohexane dump site	<i>Rhizobium</i>	<i>aggregatum</i>	Kaur et al. (2011)	USA
<i>Arabidopsis thaliana</i>	<i>Rhizobium</i>	<i>alamii</i>	Berge et al. (2009)	France
<i>Caragana intermedia</i>	<i>Rhizobium</i>	<i>alkalisoli</i>	Lu et al. (2009)	China
Activated sludge	<i>Rhizobium</i>	<i>borbori</i>	Zhang et al. (2011)	China
Sawdust of <i>Populus alba</i>	<i>Rhizobium</i>	<i>cellulosilyticum</i>	García-Fraile et al. (2007)	Spain

Table 4.1 (continued)

Host plant/place of isolation	Genus	Species	Reference	Origin
<i>Calliandra grandiflora</i>	<i>Rhizobium</i>	<i>calliandrae</i>	Rincón-Rosales et al. (2013)	Mexico
<i>Cicer arietinum</i>	<i>Rhizobium</i>	<i>ciceri</i>	Nour et al. (1994)	Spain
Cyanide treatment bioreactor	<i>Rhizobium</i>	<i>daejeonense</i>	Quan et al. (2005)	Korea
<i>Phaseolus vulgaris</i> seed-borne	<i>Rhizobium</i>	<i>endophyticum</i>	López-López et al. (2010)	Mexico
<i>Phaseolus vulgaris</i>	<i>Rhizobium</i>	<i>etli</i>	Segovia et al. (1993)	Mexico
<i>Vicia faba</i>	<i>Rhizobium</i>	<i>fabae</i>	Tian et al. (2008)	China
<i>Phaseolus vulgaris</i>	<i>Rhizobium</i>	<i>freirei</i>	Dall'Agnol et al. (2013)	Brazil
<i>Galega orientalis</i>	<i>Rhizobium</i>	<i>galegae</i>	Lindström (1989)	Finland
<i>Phaseolus vulgaris</i>	<i>Rhizobium</i>	<i>gallicum</i>	Amarger et al. (1997)	France
<i>Phaseolus vulgaris</i>	<i>Rhizobium</i>	<i>giardinii</i>	Amarger et al. (1997)	France
Tropical legumes	<i>Rhizobium</i>	<i>hainanense</i>	Chen et al. (1997)	China
<i>Dalea leporina</i> , <i>Leucaena leucocephala</i> , <i>Clitoria ternatea</i>	<i>Rhizobium</i>	<i>grahamii</i>	López-López et al. (2012)	Brazil
<i>Rosa rugosa</i> (coastal)	<i>Rhizobium</i>	<i>halophytocola</i>	Bibi et al. (2012)	Korea
Herb legumes	<i>Rhizobium</i>	<i>herbae</i>	Ren et al. (2010)	China
<i>Sesbania herbacea</i>	<i>Rhizobium</i>	<i>huautlense</i>	Wang et al. (1998)	Mexico
<i>Indigofera</i> sp.	<i>Rhizobium</i>	<i>indigoferae</i>	Wei et al. (2002)	China
<i>Calliandra grandiflora</i>	<i>Rhizobium</i>	<i>jaguaris</i>	Rincón-Rosales et al. (2013)	Mexico
Aerial tumours of <i>Ficus benjamina</i>	<i>Rhizobium</i>	<i>larrymoorei</i>	Bouzar and Jones (2001)	USA
<i>Phaseolus vulgaris</i>	<i>Rhizobium</i>	<i>leguminosarum</i>	Frank (1889)	Germany
<i>Leucaena leucocephala</i>	<i>Rhizobium</i>	<i>leucaenae</i>	Ribeiro et al. (2012)	Brazil
<i>Astragalus</i> and <i>Lespedeza</i>	<i>Rhizobium</i>	<i>loessense</i>	Wei et al. (2003)	China
<i>Lupinus</i> sp.	<i>Rhizobium</i>	<i>lupini</i>	Eckhardt et al. (1931)	
<i>Phaseolus vulgaris</i>	<i>Rhizobium</i>	<i>lusitanum</i>	Valverde et al. (2006)	Portugal
<i>Calliandra grandiflora</i>	<i>Rhizobium</i>	<i>mayense</i>	Rincón-Rosales et al. (2013)	Mexico
<i>Phaseolus vulgaris</i> , <i>siratro</i> , <i>cowpea</i> and <i>Mimosa pudica</i>	<i>Rhizobium</i>	<i>mesoamericanum</i>	López-López et al. (2012)	Mexico
<i>Lespedeza</i>	<i>Rhizobium</i>	<i>mesosinicum</i>	Lin et al. (2009)	China
<i>Medicago ruthenica</i>	<i>Rhizobium</i>	<i>miluonense</i>	Gu et al. (2008)	China
<i>Medicago ruthenica</i>	<i>Rhizobium</i>	<i>mongolense</i>	Van Berkum et al. (1998)	China
Multiple legume species native of Xinjiang	<i>Rhizobium</i>	<i>multihospitium</i>	Han et al. (2008)	China

Table 4.1 (continued)

Host plant/place of isolation	Genus	Species	Reference	Origin
Isolated from tumors on <i>Prunus cerasifera</i> , <i>Prunus avium</i> , <i>Prunus pseudocerasus</i>	<i>Rhizobium</i>	<i>nepotum</i>	Puławska et al. (2012)	Poland and Hungary
<i>Oryza alta</i>	<i>Rhizobium</i>	<i>oryzae</i>	Peng et al. (2008)	China
<i>Lemna aquinoctialis</i>	<i>Rhizobium</i>	<i>paknamense</i>	Kittiwongwattana and Thawai (2013)	Paknam district
Oil contaminated soil	<i>Rhizobium</i>	<i>petrolearium</i>	Zhang et al. (2012a)	China
<i>Phaseolus vulgaris</i>	<i>Rhizobium</i>	<i>phaseoli</i>	Dangeard (1926)	France
<i>Pisum sativum</i>	<i>Rhizobium</i>	<i>pisi</i>	Ramírez-Bahena et al. (2008)	
Rhizosphere of rice	<i>Rhizobium</i>	<i>pseudoryzae</i>	Zhang et al. (2011)	China
Rhizosphere of chickpea (<i>Cicer arietinum</i> L).	<i>Rhizobium</i>	<i>pusense</i>	Panday et al. (2011)	India
Crown gall	<i>Rhizobium</i>	<i>radiobacter</i>	Beijerinck and van Delden (1902)	The Netherlands
Hairy root disease	<i>Rhizobium</i>	<i>rhizogenes</i>	Young et al. (2001)	
Hexachlorocyclohexane dump site	<i>Rhizobium</i>	<i>rosettiformans</i>	Kaur et al. (2011)	India
Cane gall	<i>Rhizobium</i>	<i>rubi</i>	Young et al. (2001)	
Isolated from a bioreactor	<i>Rhizobium</i>	<i>selenitireducens</i>	Hunter et al. (2007)	
Tumours on chrysanthemum and cherry plu	<i>Rhizobium</i>	<i>skierniewicense</i>	Puławska et al. (2012)	Poland
Soil	<i>Rhizobium</i>	<i>solii</i>	Yoon et al. (2010)	
<i>Sphaerophysa salsula</i>	<i>Rhizobium</i>	<i>sullae</i>	Squartini et al. (2002)	China
Endolithic bacterium isolated from beach sand	<i>Rhizobium</i>	<i>subbaraonis</i>	Ramana et al. (2013)	India
<i>Kummerowia striata</i>	<i>Rhizobium</i>	<i>taibaishanense</i>	Yao et al. (2002)	China
<i>Trigonella archiducis-nicolai</i>	<i>Rhizobium</i>	<i>tibeticum</i>	Hou et al. (2009)	Tibet
<i>Sphaerophysa salsula</i>	<i>Rhizobium</i>	<i>sphaerophysae</i>	Xu et al. (2012)	China
<i>Oxytropis glabra</i>	<i>Rhizobium</i>	<i>tubonense</i>	Zhang et al. (2011)	China
<i>Neptunia natans</i>	<i>Rhizobium</i>	<i>undicola</i>	De Lajudie et al. (1998), Young et al. (2001)	Senegal
Nodules of three leguminous species	<i>Rhizobium</i>	<i>vallis</i>	Wang et al. (2013a)	China

Table 4.1 (continued)

Host plant/place of isolation	Genus	Species	Reference	Origin
<i>Vigna radiate</i>	<i>Rhizobium</i>	<i>vignae</i>	Ren et al. (2010)	China
Crown gall	<i>Rhizobium</i>	<i>vitis</i>	Ophel and Kerr (1990)	
	<i>Rhizobium</i>	<i>yanglingense</i>	Tan et al. (2001)	
<i>Kummerowia stipulacea</i>	<i>Rhizobium</i>	<i>cauense</i>	Liu et al. (2012)	
Soil	<i>Rhizobium</i>	<i>tarimense</i>	Turdahon et al. (2012)	China
<i>Oxytropis chrocephala</i>	<i>Rhizobium</i>	<i>qilianshanense</i>	Xu et al. (2012)	
<i>Acacia</i> sp.	<i>Ensifer</i>	<i>americanum</i>	Toledo et al. (2004)	
<i>Prosopis chilensis</i>	<i>Ensifer</i>	<i>arboris</i>	Nick et al. (1999)	Sudan
<i>Gycine max</i>	<i>Ensifer</i>	<i>fredii</i>	Scholla and Elkan (1984)	
<i>Acacia senegal</i> , <i>Prosopis chilensis</i>	<i>Ensifer</i>	<i>kostiense</i>	Nick et al. (1999)	Sudan
<i>Indigofera</i> sp., <i>Kummerowia stipulaceae</i>	<i>Ensifer</i>	<i>kummerowiae</i>	Wei et al. (2002)	Portugal
<i>Medicago polymorpha</i>	<i>Ensifer</i>	<i>medicae</i>	Rome et al. (1996)	
<i>Medicago</i> sp., <i>Melilotus</i> sp. and <i>Trigonella</i> sp.	<i>Ensifer</i>	<i>meliloti</i>	Dangeard (1926), De Lajudie et al. (1994)	
Bacterial predator of bacteria in soil	<i>Ensifer</i>	<i>adhaerens</i>	Wang et al. (2013a)	
<i>Sesbania canabina</i> , <i>Sesbania pachycarpa</i>	<i>Ensifer</i>	<i>saheli</i>	De Lajudie et al. (1994)	
<i>Acacia senegal</i> , <i>Prosopis juliflora</i> , <i>Acacia</i> sp., <i>Leucaena</i> sp., <i>Chamaecrista ensiformis</i>	<i>Ensifer</i>	<i>terangae</i>	De Lajudie et al. (1994)	
<i>Glycine max</i>	<i>Ensifer</i>	<i>xinjiangense</i>	Chen et al. (1988)	China
<i>Acacia angustissima</i>	<i>Ensifer</i>	<i>mexicanus</i>	Lloret et al. (2007)	Mexico
<i>Lotus arabicus</i> , <i>Lotus creticus</i> , <i>Argyrolobium uniflorum</i> and <i>Medicago sativa</i>	<i>Ensifer</i>	<i>numidicus</i>	Merabet et al. (2010)	Senegal, Tunisia
<i>Lotus arabicus</i> , <i>Lotus creticus</i> , <i>Argyrolobium uniflorum</i> and <i>Medicago sativa</i>	<i>Ensifer</i>	<i>Garamanticus</i>	Merabet et al. (2010)	Senegal, Tunisia

Table 4.1 (continued)

Host plant/place of isolation	Genus	Species	Reference	Origin
<i>Glycine max</i>	<i>Ensifer</i>	<i>sojae</i>	Li et al. (2011)	Senegal, South Africa
<i>Crotolaria species, Lotononis bainesii</i>	<i>Methylobacterium</i>	<i>nodulans</i>	Sy et al. (2001), Jaftha et al. (2002)	
<i>Listia angolensis</i>	<i>Microvirga</i>	<i>lotononidis</i>	Ardley et al. (2012)	
	<i>Microvirga</i>	<i>lupini</i>	Ardley et al. (2012)	
	<i>Microvirga</i>	<i>zambiensis</i>	Ardley et al. (2012)	Phillipines, Thailand
<i>Lupinus honoratus</i>	<i>Ochrobactrum</i>	<i>lupini</i>	Ngom et al. (2004)	
<i>Cystisus scoparius</i>	<i>Ochrobactrum</i>	<i>cytis</i>	Zurdo-Pineiroro et al. (2007)	Spain
<i>Trfolium repens, Lupinus albus</i>	<i>Phylobacterium</i>	<i>trifolii</i>	Valverde et al. (2005)	Spain
<i>Aspalathus carnosa, Cyclopia sp.</i>	<i>Burkholderia</i>	<i>tuberum</i>	Moulin et al. (2001)	South Africa
	<i>Burkholderia</i>	<i>caribensis</i>	Achouak et al. (1999)	
<i>Indigofera suffruticosa, Pithecellobium sp.</i>	<i>Burkholderia</i>	<i>contaminans</i>	Da Silva et al. (2012)	South Africa
<i>Indigofera suffruticosa, Pithecellobium sp.</i>	<i>Burkholderia</i>	<i>fungorum</i>	Da Silva et al. (2012)	
<i>Indigofera suffruticosa, Pithecellobium sp.</i>	<i>Burkholderia</i>	<i>lata</i>	Da Silva et al. (2012)	South Africa
<i>Mimosa pigra, Mimosa scabrella</i>	<i>Burkholderia</i>	<i>mimosarum</i>	Chen et al. (2006)	
<i>Mimosa bimucronata, Mimosa scabrella</i>	<i>Burkholderia</i>	<i>nodosa</i>	Chen et al. (2007)	South Africa
<i>Machaerium lunatum, Mimosa invisa</i>	<i>Burkholderia</i>	<i>phymatum</i>	Vandamme et al. (2002), Elliott et al. (2007b)	
<i>Mimosa caesalpiniiifolia</i>	<i>Burkholderia</i>	<i>sabiae</i>	Chen et al. (2008)	South Africa
	<i>Burkholderia</i>	<i>symbiotica</i>	Sheu et al. (2012)	
<i>Mimosa sp.</i>	<i>Burkholderia</i>	<i>diazotrophica</i>	Sheu et al. (2012)	South Africa
<i>Mimosa pudica</i>	<i>Cupriavidus</i>	<i>taiwanensis</i>	Van Damme and Coenye (2004)	
<i>Mimosa sp.</i>	<i>Cupriavidus</i>	<i>necator</i>	Makkar and Casida (1987)	Japan
	<i>Cupriavidus</i>	<i>numazuensis</i>	Martínez-Aguilar et al. (2012)	
	<i>Cupriavidus</i>	<i>oxalaticus</i>	Sahin et al. (2000), Vandamme and Coenye (2004)	Argentina
Soil from pampas	<i>Cupriavidus</i>	<i>pampae</i>	Vandamme et al. (1999), Vandamme and Coenye (2004)	
Water	<i>Cupriavidus</i>	<i>pauculus</i>		

Table 4.1 (continued)

Host plant/place of isolation	Genus	Species	Reference	Origin
Volcanic mudflow deposits from Mt. Pinatubo	<i>Cupriavidus</i>	<i>pinatubonensis</i>	Sato et al. (2006)	Phillippines
Respiratory tract of cystic fibrosis patients	<i>Cupriavidus</i>	<i>respiraculi</i>	Coenye and Vandamme (2003), Vandamme and Coenye (2004)	
<i>Kummerowia stipulacea</i>	<i>Shinella</i>	<i>kummerowiae</i>	Lin et al. (2008)	China
Soil	<i>Shinella</i>	<i>granuli</i>	An et al. (2006)	China
Soil	<i>Shinella</i>	<i>yambaruensis</i>	Matsui et al. (2009)	Japan
Domestic waste compost	<i>Shinella</i>	<i>fusca</i>	Vaz-Moreira et al. (2010)	
Sludge of a leach-ate treatment plant	<i>Shinella</i>	<i>daejeonensis</i>	Lee et al. (2011)	Korea
<i>Phaseolus vulgaris</i>	<i>Herbaspirillum</i>	<i>lusitanum</i>	Valverde et al. (2003)	Portugal

Recently, a new species, *A. doeberienerae*, was isolated from *Sesbania virgata* (Moreira et al. 2006). *Azorhizobium* deserves its status as a separate genus based on many distinct properties including its SSU sequence (Young and Haukka 1996).

4.3.2 *Bradyrhizobium*

Until recently, all *Bradyrhizobium* strains that nodulate soybean were known as *Bradyrhizobium japonicum* (Jordan 1982), though it had long been recognised that they were too diverse to fit in one species. The genus *Bradyrhizobium* is made up of slow-growing bacteria that produce an alkali reaction on BTB media (Vincent 1970). Currently, eight species namely: *B. japonicum* (Jordan 1982), *B. elkanii* (Kuykendall et al. 1992), *B. liaoningense* which is distinct by DNA–DNA, besides having a very slow growth rate (Xu et al. 1995), *B. canariense* (Vinueza et al. 2005), *B. jicamae* (Ramirez-Bahena et al. 2008), *B. yuanmingense* (Appunu et al. 2009), *B. iriomotense* (Islam et al. 2010), with the last species, *B. betae*, not able to form root-nodules on *Beta vulgaris* (original host) as well as on *Glycine max* and *Pachyrhizus ahipa*. There are many strains, isolated from tropical legumes such as cowpea that belong to *Bradyrhizobium*, but do not nodulate soybean. These are currently assigned *Bradyrhizobium* sp. but would have to be assigned different species eventually based on genetic differences and similarities. These *Bradyrhizobium* sp. have diverse hosts including cowpea, acacias, soybean, and bambara groundnut with the rest listed in Table 4.1.

Based on the number of identified species (18) in this genus (Tables 4.1 and 4.2), it appears as though *Bradyrhizobium* is not a very diverse group compared to for

example *Rhizobium*. Many species of this genus isolated from Africa are assigned *Bradyrhizobium* sp. a not so defined group. It appears as if, bradyrhizobia have a very wide host range from grain legumes such as cowpea and bambara groundnut (Krasova-Wade et al. 2003; Pule-Meulenberg and Dakora 2010; Pule-Meulenberg et al. 2011), herbaceous legumes such as *Zornia glochidiata* (Gueye et al. 1990), and *Acacia* sp. (Odee et al. 1997). The fact that there are many *Bradyrhizobium* sp. that are not fully defined seems to characterise this genus (Sprent 2009). Many such incompletely defined bradyrhizobia falls into the so called cowpea miscellany. Given the large diversity of legumes particularly in semi-arid Africa, one would expect an equally diverse population of microsymbiont partners. A possible reason could be that there is not much research undertaken in this area especially since very few African governments sponsor research. Despite the large number of strains isolated through much scientific exploration, the number of named species under the genus *Bradyrhizobium* is still low.

4.3.3 *Mesorhizobium*

It had long been recognised that *Rhizobium loti* was different from other fast-growing species such *R. leguminosarum* and *Rhizobium meliloti* based on its SSU sequence (Young and Haukka 1996). Jarvis et al. (1997) proposed the establishment of a new genus *Mesorhizobium* and suggested the transfer of *R. loti*, *R. huakuii*, *R. ciceri*, *R. mediterraneum* and *R. tianshanense* to the new genus. Since then, the new genus grew vastly and currently has 30 known members (this review). In addition to the above species, this genus currently consists of *M. albiziae*, nodulating *Albizia kalkora*, *A. julibrissin*, *Glycine max*, *Leucaena leucocephala* and *Phaseolus vulgaris* (Wang et al. 2007); *M. alhagi*, nodulating *Alhagi sparsifolia*, *Sophora alopecuroides*, *Glycyrrhiza inflata*, *Medicago sativa*, *Indigofera endecaphylla*, *Vicia cracca* and *Sophora flavescens* (Chen et al. 2011); *M. amorphae*, nodulating *Amorpha fruticosa* and *Cicer arietinum* (Wang et al. 1999; Rivas et al. 2007); *M. australicum* and *M. opportunistum*, nodulating *Bisserula pelecinus*, *Astragalus membranaceus* and *siratiro* (Nandasena et al. 2009), with *M. opportunistum* also forming nodules on *Lotus peregrinus*; *M. camelthorni*, nodulating *Alhagi sparsifolia*, *Sophora alopecuroides*, *Glycyrrhiza inflata* and *Medicago sativa* (Chen et al. 2011); *M. caraganae*, nodulating *Caragana microphylla* and *Caragana intermedia* (Guan et al. 2008); *M. chacoense*, nodulating *Prosopis alba* (Velázquez et al. 2001); *M. gobiense*, nodulating *Astragalus filicaulis*, *Lotus frondosus*, *Lotus tenuis* and *Oxotrophis glabra* (Han et al. 2008); *M. metallidurans*, nodulating *Anthyllis vulneraria* (Vidal et al. 2009); *M. plurifarum*, nodulating *Acacia* sp. *Leucaena* sp. *Prosopis juliflora* and *Chamaecrista ensiformis* (De Lajudie et al. 1998); *M. robiniae*, nodulating *Robinia pseudoacacia* (Zhou et al. 2010); *M. septentrionale*, nodulating *Astragalus adsurgens*, *P. vulgaris*, *G. max*, *L. leucocephala*, *M. atropurpureum*, *Lotus corniculatus* and *Robinia pseudoacacia* (Gao et al. 2004); *M. shangrilense*, nodulating *Caragana* sp. *Glycyrrhiza uralensis*, *Astragalus adsurgens*, *Vigna unguiculata*, *Vigna radiata* and *P. vulgaris* (Lu et al. 2009b); *M. tarimense*, nodulating *Lotus*

frondosus and *Lotus tenuis* (Han et al. 2008); *M. temperatum* nodulating *Astragalus adsurgens*, *P. vulgaris*, *V. unguiculata*, *G. max*, *L. leucocephala*, *M. sativa* and *Lotus corniculatus* (Gao et al. 2004) and *M. thiogangeticum*, isolated from *Clitoria ternatea* although it does not nodulate this host (Ghosh and Roy 2006). Since the last compilation by Lindström et al. (2010), eight new species namely *M. silamurunense*, *M. muleiense*, *M. abyssinicae*, *M. hawassense*, *M. shonense*, *M. qingshegii* and *M. sangaii* have been described (Degefu et al. 2012; Ramírez-Bahena et al. 2012; Zhang et al. 2012; Zhou et al. 2013).

4.3.4 *Rhizobium*

The genus *Rhizobium* is by far the largest and most diverse with 63 species, but 12 of them lacking the ability to nodulate legumes. The later include all former *Agrobacterium* species which have since been incorporated into *Rhizobium* genus. It is also growing very fast, with about 9 new members described in the past two years. Other changes in the genus include the naming of *Rhizobium tropici* type A strain into a new species *R. leucaena* based on 16S rRNA gene sequences and DNA–DNA hybridization (Martínez-Romero et al. 1991; Ribeiro et al. 2012). Chen et al. (1997) reported a new rhizobium species on the basis of 16S rRNA gene sequence, DNA–DNA hybridization and phenotypic characterization, this new species belong to the phylogenetic tree branch which included *R. leguminosarum*.

Like with other genera, many of the new additions to this genus come from China. For those that are fixing-N₂, the homologous hosts belong to the legume tribe Papilionoideae with *Chamaecrista ensiformis* as one of the few Caesalpinioideae. The non-nodulating ones have been isolated in somewhat odd places. For example, *R. daejeonense* was isolated from a cyanide treatment bioreactor (Quan et al. 2005), *R. aggregatum* from a hexachlorocyclohexane dump site (Kaur et al. 2011) and so on (see Table 4.1). Interestingly, some nodulating bacteria have been isolated from unusual habitat e.g., termite gut. It is also interesting that the diversity of the genus is seemingly high in the temperate regions, although some areas of China where some of the isolation took place are arid to semi-arid. However, other studies, particularly in semi-arid areas have isolated fast-growing rhizobia (Pule-Meulenberg and Dakora 2010).

4.3.5 *Ensifer*

Not so long ago, Willems et al. (2003) requested an opinion of the Judicial Commission of the International Committee on Systematics of Prokaryotes with a proposal that the name *Sinorhizobium* should be preferred to *Ensifer*. The reasons for such a request were that: *Sinorhizobium* was much well-known compared to *Ensifer*; that the descriptive name derived from ‘rhizobium’ implied distinctive features of the organisms, and *Ensifer* had only one species while *Sinorhizobium* had nine species

(Young 2003). The Committee ruling was that *Ensifer* had priority over *Sinorhizobium*, and hence all species of *Sinorhizobium* were transferred to *Ensifer*. Currently, there are 15 species in this genus, including *E. adhaerens* (Willems et al. 2003, Merabet et al. 2010), *E. arboris* and *E. kostiense* (Nick et al. 1999), *E. fredii* (Chen et al. 1988), *E. garamanticus* (Merabet et al. 2010), *E. kummerowia* (Wei et al. 2002), *E. meliloti* (Dangeard 1926; Rome et al. 1996; Yan et al. 2000), *E. medicae* (Rome et al. 1996), *E. mexicanus* (Lloret et al. 2007), *E. morelense* (Wan et al. 2002), *E. numidicus* (Merabet et al. 2010), *E. saheli* (De Lajudie et al. 1994), *E. terangaie* (De Lajudie et al. 1994) and *E. xinjiangense* (Chen et al. 1988). Merabet et al. (2010) have added the latest members of this genus namely *E. garamanticus* and *E. numidicus* isolated from root nodules of *Lotus arabicus* in Senegal and *Lotus creticus*, *Argyrobium uniflorum* and *Medicago sativa* in Tunisia.

The last three known members of this genus are *S. americanum*, which nodulates *Acacia* sp. (Toledo et al. 2003), *S. abris*, nodulating *Abrus precatorius* and *S. indiane*, nodulating *Sesbania rostrata* (Ogasawara et al. 2003). Although they belong to the *Ensifer* phylogenetic cluster, these last three species were described before the transfer of *Sinorhizobium* to *Ensifer* (Young 2003).

4.3.6 *Methylobacterium*

Methylobacterium nodulans, isolated from root-nodules of *Crotalaria* species (Sy et al. 2001; Jourand et al. 2004) and *Lotononis bainesii* (Jaftha et al. 2002) is still the only known species that nodulates this host (Sprent 2009). Its main characteristic is the ability to metabolise methanol, as another species nodulating some *Lotononis* species does not (Yates et al. 2007; Sprent 2009). It is suspected that *Methylobacterium nodulans* acquired its nodulation genes by lateral gene transfer.

4.3.7 *Burkholderia* and *Cupriavidus*

To date, eleven species, from three genera have been reported to form nodules on several host plants (Lindström et al. 2010). There seem to be a strong relation between nodulating species and pathogens. The first species to be properly identified *Cupriavidus taiwanensis* (then called *Ralstonia taiwanensis*), was isolated from nodules (Chen et al. 2001), but also from sputum of a cystic fibrosis patient (Sprent 2009). The genus, *Burkholderia* also has species that are plant and animal pathogens as well as legume symbionts. The latest information indicates that the disease causing and symbiotic *Burkholderia* belong to two distinct lineages (Angus et al. 2013). They came to this conclusion after analysis of the 16S rRNA and other gene phylogenies as well as testing, examining the genomes (*Burkholderia*) for virulence as well as performing functional tests for pathogenicity. So far, seven species of *Burkholderia* are known to nodulate legumes, and these include *B. caribensis*, nodulating *Mimosa pudica* and *Mimosa diplotricha* (Vandamme et al. 2002; Chen

et al. 2003); *B. mimosarum*, nodulating *Mimosa pigra*, *Mimosa scabrella* (Chen et al. 2006); *B. nodosa*, nodulating *Mimosa bimucronata* and *Mimosa scabrella* (Chen et al. 2007); *B. phymatum*, nodulating *Machaerium lunatum* and *Mimosa invisa* (Vandamme et al. 2002; Elliott et al. 2007b); *B. sabiae*, nodulating *Mimosa caesalpiniiifolia* (Chen et al. 2008), *B. tuberum*, nodulating *Aspalathus carnosa*, *Cyclopia* sp. siratro (Vandamme et al. 2002; Elliott et al. 2007a) and so on (Table 4.1). In addition, there have been reports of *Burkholderia* nodulating *Lebeckia* (F.L. Phalan, personal communication), *Psoralea* sp. (Kanu and Dakora 2012). Lastly, a new *Herbaspirillum lusitanum* was found to nodulate *P. vulgaris* (Valverde et al. 2003).

4.3.8 *Ochrobactrum*

This genus is classified in the family Brucellaceae, which includes important animal and human pathogenic genus *Brucella*. As with β rhizobia, there are similarities between nodulating bacteria and pathogens. Ngom et al. (2004) were the first to report on a species of *Ochrobactrum* that formed effective nodules on *Acacia mangium*, *Faidherbia albida* and *Falcataria moluccana*, all mimosoid legumes. The second report was of *O. lupini*, isolated from *Lupinus honoratus* in Argentina and was able to nodulate *Lupinus albus* (Sprenst 2009). The latest report was that of *Ochrobactrum cystis*, nodulating *Cystisus scoparius* (Zurdo-Pineiro et al. 2007).

4.3.9 *Shinella*

Shinella is a genus of the family Rhizobiaceae. The first report of a member of this genus forming N_2 -fixing nodules on a legume was by Lin et al. (2008) who isolated *Shinella kummerowiae* from root nodules of *Kummerowia stipulacea* in Shandong province of China. The *nifH*, *nodC* and *nodD* sequences of their isolate were very similar to those of bean nodulating *Rhizobium tropici* strains (now called *R. leucaena*). Other members of this genus include *S. granuli* (An et al. 2006) isolated from soil in China, *S. yambaruensis* (Matsui et al. 2009) obtained from soil in Japan, *S. fusca* (Vaz-Moreira et al. 2010), isolated from domestic waste compost and *S. daejeonensis* isolated from a sludge of a leachate treatment plant in Korea. *Shinella kummerowiae* is the only member known to nodulate a legume plant.

4.3.10 *Achromobacter xylosoxidans*

It is a Gram-negative and catalase positive bacteria which has been frequently retrieved in rhizosphere, as a natural endophytes of wheat (Jha and Kumar 2009), from disease suppressive soil as a PGPR. Tilak et al. (2005) suggested that *A.*

xylosoxidans has natural ability to tolerate salinity because it was identified in a bacterial community isolated from the rhizosphere of salt-affected rice.

Some actinobacteria such as *Streptomyces lydicus* were reported to colonize pea nodules (Tokala et al. 2002); Bai et al. (2003) showed that *Bacillus subtilis* and *Bacillus thuringiensis* can naturally co-inhabit soybean nodules along with *B. japonicum*.

4.4 Rhizobial Symbionts from African Soils

African soils harbor a large diversity of indigenous rhizobial symbionts and other bacterial endophytes. This fact can be regarded as either a blessing or a curse. It can be a good thing because there is a large population from where to select elite strains with potential to be developed into inoculant to be used as biofertilizer. The high indigenous resident microbial population may present a challenge with regard to competition with the inoculant strains. A competitive rhizobial strain is one that is successful in nodule occupancy (Bogino et al. 2008). According to Sprent (2009), there are many aspects to this, including ability to persist in the soil in the absence of a host, to grow actively and attach to roots. Pule-Meulenberg and Dakora (2010) ascertained that where there was sole occupancy by a strain IGS type (that is, highly competitive), the symbiotic efficiency was enhanced. In another experiment where the promiscuous *Glycine max* (soybean) TGx varieties bred by the International Institute of Tropical Agriculture (IITA) to nodulate with indigenous rhizobial populations on African soils, it was shown that the inoculant strain, *B. japonicum* WB74 was outcompeted by some indigenous strains in some varieties (Pule-Meulenberg and Dakora 2010). They also showed that one of the varieties TGx 1445–3E could derive more than 60% of its N from symbiotic fixation and could derive about 100 kg N/ha without inoculation.

However, the available literature (Singleton and Tavares 1986; Thies et al. 1991) revealed the indigenous rhizobial population as a nuisance because often they out-compete the inoculant strain. For instance, Mpeperekhi et al. (2000) demonstrated that TGx varieties produced erratic nodulation and N₂ fixation in Zimbabwe and Zambian soils, suggesting negative competition amongst the indigenous rhizobial population. Despite this, African soils still harbor a highly diverse population of microsymbionts that are good candidates for inoculant strains because many African soils are centers of origin for many plants. Therefore, it is expected that such gene centers would have diverse microorganisms associated with the legumes (Vavilov 1926). For example, Botswana is considered to be the centre of diversity for the wild cowpea species (Mazhani 1995) and hence, these plants are expected to have diverse effective microsymbiont partners in Botswana and in similar semi-arid to arid environments. However, in most semi-arid areas in Africa, research on diversity of rhizobial bacteria for legumes is still at its infancy. It is evident from Table 4.2 that very few studies have been conducted in Africa in comparison to Table 4.1 which gives a global picture.

Table 4.2 Diversity of rhizobia nodulating some legume species in semi-arid Africa

Legume species	Associated rhizobial partner	Origin	Reference
<i>Crotolaria</i> species	<i>Methylobacterium nodulans</i>	Senegal	Sy et al. (2001)
<i>Lotononis bainesii</i>	<i>Methylobacterium nodulans</i>	South Africa	Jaftha et al. (2002)
<i>Aspalathus carnosa</i>	<i>Burkholderia tuberum</i>	South Africa	Moulin et al. (2001)
<i>Cyclopia</i> sp.	<i>Burkholderia tuberum</i>	South Africa	Elliott et al. (2007a)
<i>Lebeckia</i> sp.	<i>Burkholderia</i> sp.	South Africa	Beukes et al. (2013)
<i>Psoralea</i> sp.	<i>Burkholderia</i> sp.	South Africa	Kanu and Dakora (2012)
<i>Sesbania rostrata</i> , <i>Sesbania</i> sp., <i>Acacia laeta</i>	<i>Sinorhizobium terangae</i>	Senegal	De Lajudie et al. (1994)
<i>Sesbania canabina</i>	<i>Sinorhizobium sahel</i>	Senegal	De Lajudie et al. (1994)
<i>Sesbania pachycarpa</i>			
<i>Acacia senegal</i> , <i>Prosopis juliflora</i> , <i>Acacia</i> sp., <i>Leucaena</i> sp., <i>Chamaecrista ensiformis</i>	<i>Mesorhizobium plurifarum</i>	Senegal	De Lajudie et al. (1998)
<i>Pterocarpus erinaceus</i> , <i>Pterocarpus lucens</i>	<i>Rhizobium</i> sp., <i>Mesorhizobium</i> species	Senegal	Sylla et al. (2002)
<i>Zornia glochidiata</i>	<i>Bradyrhizobium</i> sp.	Senegal	Gueye et al. (1990)
<i>Vigna unguiculata</i>	<i>Bradyrhizobium</i> sp.	Botswana, South Africa, Ghana	Pule-Meulenberg et al. (2010)
<i>Vigna unguiculata</i>	<i>Bradyrhizobium</i> sp.	Senegal	Krasova-Wade and Neyra (2007)
<i>Glycine max</i>	<i>Bradyrhizobium</i> sp.	Ghana	Pule-Meulenberg et al. (2011)
<i>Sesbania rostrata</i>	<i>Azorhizobium caulinodans</i>	Senegal	Dreyfus et al. (1988)
<i>Galegae orientalis</i>	<i>Rhizobium galegae</i>	Ethiopia	Lindström (1989)

The most important legume crops grown by farmers in the semi-arid areas of Africa are cowpea, *Vigna unguiculata*, Bambara groundnut and *Arachis hypogea* in the order of importance (Azam-Ali et al. 2001). A number of studies have assessed nitrogen fixation in cowpea (Belane and Dakora 2009; Makoi et al. 2009; Naab et al. 2009; Pule-Meulenberg and Dakora 2010; Yakubu et al. 2010) in the semi-arid areas of Ghana, Botswana and South Africa. Fewer studies have evaluated the diversity of the root-nodule bacteria associated with cowpea (Krasova-Wade et al. 2003; Steenkamp et al. 2008; Pule-Meulenberg and Dakora 2010; Mathu et al. 2012) and competition aspects (Eaglesham et al. 1981; Bloem and Law 2001). Even fewer studies are on Bambara groundnut, whether the N_2 fixation of the crop (Nyemba and Dakora 2010), or the associated rhizobia. It is able to grow under very nutrient poor soil conditions and very little rainfall compared to other crops (Doku and Karikari 1971). Table 4.2 presents some of nodulated legumes and the associated microsymbiont partners isolated from their root nodules from semi-arid parts of Africa. From this table it is evident that isolation of rhizobia from root nodules of

legumes growing in semi-arid areas does not happen often despite the rich diverse plants and their associated rhizobial population.

4.5 Conclusion

Many investigations on rhizobia are aimed at studying them as organisms that have the ability to fix N_2 symbiotically. The majority of such studies were carried out in the past 15 years, most probably due to adoption of new molecular tools that are able to resolve bacteria at a species and genospecies levels. The 16S rDNA played an important role in this, although a number of reports caution its inability to differentiate between closely related organisms (Laguerre et al. 1997). It is noteworthy that the latest discoveries of new rhizobial species are coming out of Asia, specifically China. This is to be expected because it is a large country with many different soil ecologies that are likely to harbour different types of microorganisms. Although the semi-arid and arid areas are many in Africa, they are part of many countries and research is not coordinated. Also, very few studies have investigated the diverse indigenous legumes and their associated microsymbionts. Isolation of rhizobia from diverse legumes and habitats is likely to uncover novel and superior strains that can be used for inoculum development.

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Chapter 5

Genetic Diversity of Soybean Root Nodulating Bacteria

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Abstract Soybean (*Glycine max* L. Merrill), is a summer annual herb grown extensively in China, Argentina, Brazil and USA. It was thought to be very selective for its symbiotic partner. Earlier, only *Bradyrhizobium japonicum* was reported to nodulate soybean. It now is reported to be nodulated by a number of rhizobial genera and species; *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium* and *Sinorhizobium*. *Sinorhizobial* species (*fredii*) nodulating soybean shows broad host range, where as the slow growing *Bradyrhizobium* is more selective. Slow growing *B. japonicum*, *B. elkanii*, *B. liaoningense* and *B. yuanmingense* are more effective nodulators of soybean. Fast growers *Rhizobium tropici*, *Rhizobium* sp., *Rhizobium oryzae* and *Mesorhizobium tianshanense* have also been reported to form nodules on soybean. A large genetic diversity exists within the slow growing *Bradyrhizobium* isolates nodulating soybean. Due to the ecological and economic importance, the *Bradyrhizobia* species and their diversity have been extensively investigated in the last few years. The diversity and the size of indigenous population in soil can vary with the presence of the host legume and the history of the land use pattern at the sampling site. This review focuses on the genetic diversity existing in the bacteria nodulating soybean with special reference to Indian work.

5.1 Introduction

The rhizobium–legume symbiosis has been widely studied as the model of mutualistic evolution and the essential component of sustainable agriculture as biological fixation is the main source of nitrogen for natural and agricultural ecosystems. In agriculture, the symbiosis of nitrogen-fixing bacteria, collectively known as rhizobia, with crops belonging to the family Leguminosae (Fabaceae) are the most studied. Relatively high contributions to nitrogen nutrition have been demonstrated in pulses, fodders, green manures and trees. Members of the genus *Bradyrhizobium* constitute an important group of rhizobia, some of which form symbioses with economically important crops, such as soybean [*Glycine max* (L.) Merr.].

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Soybean (*Glycine max* L. Merr) is an annual herbaceous plant with its probable centre of origin and domestication in East Asia (Gai et al. 2000; Xu et al. 2002; Abe et al. 2003; Zhao and Gai 2004). For centuries soybean has been grown in China, Japan, Korea, Manchuria, the Philippines and Indonesia for various purposes and has been lovingly referred to as ‘Cow of the field’, or ‘Gold from soil’. India is the fifth largest producer of soybean in the world next to United States, Brazil, Argentina and China. In India, Madhya Pradesh also known as the ‘Soy State’ alone contributes 56–67% in total area and production of soybean. About 97% of total area and 96% production of soybean in the country come from the three states of Madhya Pradesh, Maharashtra and Rajasthan.

Soybean is a major source of vegetable oil, protein, animal feed and is also used for improving soil nutrient status. It is the seventh most harvest crops of the world by tonnage. Soybean alone is estimated to produce up to 200 kg N ha⁻¹ in aboveground biomass in a single growing season. Of the soybean nitrogen content, 58–68% is estimated to be derived from symbiotic nitrogen fixation (Salvagiotti et al. 2008; Peoples et al. 2009; Jensen et al. 2012). Following harvesting, the remaining portions of the plant, including roots and nodules which represent 30–60% of the nitrogen content, are left to replenish the nitrogen content of the surrounding soil. Soybean contains about 22% essential amino acids, over 40% proteins and about 22% oil. Soybean oil contains about 85% unsaturated fatty acids (Malik et al. 2006) making it among the lowest of the vegetable oils. It is also rich in Omega 3 and Omega 6 fatty acids essential for regulating lipid and cholesterol metabolism. Soybean is reported to have several health benefits such as lowering cholesterol (Anderson et al. 1995; Van Horn et al. 2008), suitable dietary option – for type 2 diabetes due to its mostly starch free carbohydrates (Holt et al. 1996) and for the prevention and control of obesity (Velasquez and Bhathena 2007).

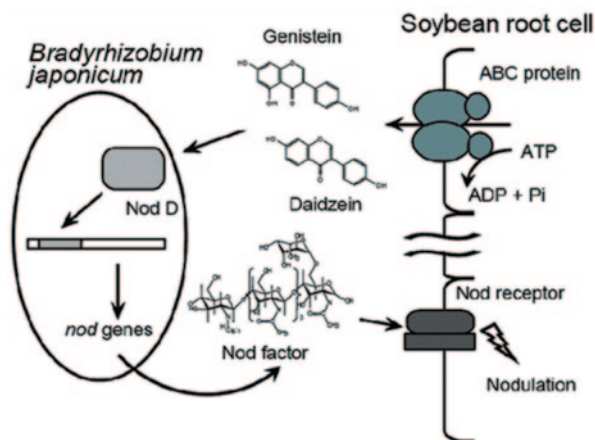
5.2 Soybean—*Bradyrhizobium* Symbiosis

Like any other legume soybean forms symbiotic partnership with Gram-negative α -Proteobacterial members, viz., *Sinorhizobium* (*Ensifer*) and *Bradyrhizobium*. Soybean forms determinate nodules which lose meristematic activity shortly after initiation, thus growth is due to cell expansion resulting in mature nodules which are spherical in shape. When Fred et al. (1932) proposed seven cross inoculation groups for categorizing root nodule bacteria, soybean was found to nodulate with only one genus and species; *Rhizobium japonicum*. However, in time it was observed that the legume could form effective symbiotic relationship with more genera and species.

5.2.1 Root Exudates and Molecular Signaling

The inter-recognition via chemical signals between a host plant and rhizobium is highly specific for both species and is indispensable for the development of

Fig. 5.1 A model of flavonoid secretion from soybean roots and the inter-recognition between legume and rhizobium. (Sugiyama et al. 2008)



functional nodules. These sophisticated signaling cascades begin with the secretion of flavonoids from the roots of the host plant, which are recognized by the NodD protein in the rhizobia leading to the successive induction of *nod* gene expression to produce the second signaling molecules, lipochitooligosaccharides, also known as Nod-factors (Fig. 5.1) (Sugiyama et al. 2008). Nod-factors perceived by receptors in the host plant induce various signaling events such as ‘calcium spiking’ response, ultimately resulting in the formation of nodules. As signaling flavonoids, isoliquiritigenin, genistein and daidzein were identified in root exudates of soybean (Kosslak et al. 1987; Smit et al. 1992). However, it is still not clear how the same host genotype producing these two specific flavonoids genistein and daidzein can recruit different rhizobial genera to form effective partnership. Obviously, there must be something common produced by the rhizobial species which is the main recognition factor for the flavonoid secreted by the plant. However, comparative genomics of *Sinorhizobium fredii* and *B. japonicum* hasn’t shown any gene common or specific to these soybean microsymbionts compared with other legume microsymbionts. This shows that there is no gene specifically shared by *Sinorhizobium* and *Bradyrhizobium* to establish symbiosis with soybean (Tian et al. 2012).

5.2.2 Nodule Development

Nodulation involves the intimate relationship of *Bradyrhizobium/Sinorhizobium* and soybean plants, which results in the formation of a novel organ, the nodule, in which the bacteria reside and provide a steady source of nitrogen to the plant. Nodule formation requires nodule organogenesis and bacterial infection which must be both spatially and temporally coordinated. The two processes requires multiple and complex chemical cross-talk and coordinated expression of several genes (Oldroyd et al. 2011). Primarily through an examination of plant mutants defective in nodulation, the basic steps in the Nod factor signaling pathway have been elucidated

(Oldroyd and Downie 2008). Initial Nod factor recognition is mediated through two LysM receptors that likely directly interact with the compatible Nod factor (Amor et al. 2003; Madsen et al. 2003; Radutoiu et al. 2003). The resulting signaling cascade ultimately leads to activation of specific transcription factors (TFs), a few of which have been identified.

Perception of Nod factors by the receptors GmNFR1 and GmNFR5 in soybean roots (Indrasumunar et al. 2010, 2011) triggers many of the early events in nodulation including ion fluxes, root hair curling and root cortical cell division (Denarie et al. 1996; Kamst et al. 1998; Hirsch and Oldroyd 2009). The recognition of nod factors leads to nucleolar calcium spiking and the subsequent activation of the symbiotic signaling pathway which requires symbiosis receptor-like kinase (SYMRK) (Endre et al. 2002). Calmodulin dependent protein kinase (CCaMK) (Levy et al. 2004; Mitra et al. 2004; Tirichine et al. 2006) and a protein of unknown function (CYCLOPS) (Yano et al. 2008) are needed for perception of calcium spiking. This further initiates downstream signaling events including expressions of several transcription factors (nodulation signaling pathway [NSP]1, NSP2, ERF required for nodulation [ERN]1, nodule inception [NIN]) that regulate NF-induced gene expression and subsequently leading to nodule morphogenesis (Schauser et al. 1999; Kalo et al. 2005; Smit et al. 2005; Andriankaja et al. 2007; Marsh et al. 2007; Oldroyd et al. 2011). Soybean root nodules are of the 'determinate' type as they arise from the central cortex and have a transient meristem (Ferguson et al. 2010). Typically, soybean nodules senesce after few weeks and they are eventually replaced by developing new nodule structures.

In order to limit loss of carbon through excessive nodulation and also to maintain the balance in the symbiosis association, nodulation in soybean is tightly regulated through the Autoregulation of Nodulation (AON) pathway. The AON pathway operates through long distance signaling between the root and shoot. In soybean, GmRIC1 and GmRIC2 which are CLV3/ESR-related (CLE) peptide hormones (Lim et al. 2011; Reid et al. 2011; Reid et al. 2013) are synthesized in the roots and then translocated to the shoots via xylem (Delves et al. 1986; Reid et al. 2011) where they are recognized by the LRR receptor kinase, GmNARK/LjHAR1/MtSUNN/PsSYM29 (Krusell et al. 2002; Searle et al. 2003; van Hameren et al. 2013). In soybean, kinase-associated protein phosphatases, *GmKAPP1* and *GmKAPP2* are believed to be working in conjunction with the LRR receptor kinase, GmNARK in the signal transduction pathway (Miyahara et al. 2008; Ferguson et al. 2010). Shoot-Derived Inhibitor (SDI) is then produced and translocated to the root via the phloem upon perception of the CLE signal which eventually inhibits further nodulation (Ferguson et al. 2010; Reid et al. 2011). Several other genes including *LjKLAVIER* (Oka-Kira et al. 2005), ERF transcription factor, *MtEFD* (Vernie et al. 2008), and *LjASTRAY* (Nishimura et al. 2002) are known to regulate nodule numbers. Nodulation is also regulated by various environmental stresses such as acidity and soil nitrate (Carroll et al. 1985; Lin et al. 2012). All in all nodule development is regulated both by the genetic makeup of the host as well as environmental cues.

5.3 Genetic Diversity of Root Nodule Symbionts

The most important factor of nodule formation is the prokaryotic symbiont. Soybean (*Glycine max*) is known to be nodulated by at least five species of rhizobia belonging to two different genera with almost all the species reported from China. These species includes both the slow growing and fast growing rhizobia with diverse morphological, biochemical, physiological, genetic and symbiotic attributes. Generally, fast growing rhizobia are classified as those bacteria having generation of about 1.5–4 h and produces acid on YEM (Yeast Mannitol Medium). Slow growing bradyrhizobia on the other hand, have a generation time of at least 6 h and produces alkali on YEM medium (Table 5.1). *Bradyrhizobium japonicum*, *B. elkanii*, *B. liaoningense*, *B. yuanmingense* are the slow growing rhizobia nodulating soybean and *S. fredii* the fast growing symbiont (Man et al. 2008; Vinuesa et al. 2008; Han et al. 2009; Li et al. 2011a, b; Zhang et al. 2011). Clear biogeography of soybean rhizobia has been shown in China (Han et al. 2009; Man et al. 2008; Wang et al. 2009). However, less is known about soybean's nodule microsymbionts in an ecological context despite its long history of cultivation and wide distribution across Indian continent.

5.3.1 Fast Growing Isolates

The characterization of the indigenous fast-growing symbionts of soybean root nodules is important to understand better the ecological strategies that tropical rhizobia may take to survive after the introduction of an exotic host legume. In (1982) fast-growing rhizobial strains were isolated from soybean nodules and from soil of the People's Republic of China, within the center of origin and diversity of this legume (Keyser et al. 1982). Later, fast-growing strains were isolated from other primary and secondary centers of soybean origin (Xu and Ge 1984; Dowdle and Bohloul 1985; Rodriguez-Navarro et al. 1996). These fast growers were classified as the new species *Rhizobium fredii* (Scholla and Elkan 1984), later reclassified as *S. fredii* and *S. xinjiangensis* (Chen et al. 1988), and recently proposed to change to the genus *Ensifer* (Young 2003). Although it was originally thought that *S. (Ensifer) fredii* was specific for Asian soybean lines (Keyser et al. 1982; Stowers and Eaglesham 1984), later it has been shown that several North American and Brazilian genotypes are capable of forming effective nodules with those bacteria (Balatti and Pueppke 1992; Chueire and Hungria 1997). Based upon phenotypic characterization and DNA–DNA hybridization (Chen et al. 1988; Peng et al. 2002), two species of fast growers *S. fredii* and *S. xinjiangensis* were proposed, however, *E. xinjiangensis* has subsequently been incorporated into *S. fredii* based upon multilocus sequence analysis (Martens et al. 2008).

In recent times genetic diversity in *S. fredii* strains have been observed by different workers. Their work has categorized fast growing strains nodulating *G. max* into five genomic groups; *S. fredii*, *Sinorhizobium* sp. I, *Sinorhizobium* sp. II,

Table 5.1 Diversity of root nodulating bacteria isolated from soybean

Strains	Generation time	Geographic origin of strains	Colony size (mm)	G+C (%)	References
<i>Bradyrhizobium japonicum</i>	>6 h	Ibaraki, Japan	1.0 ^c	61–65	Jordan (1982)
<i>B. elkanii</i>	>6 h	China	1.0 ^c	62–64	Kuykendall et al. (1992)
<i>B. liaoningense</i>	16.4–39.6 h	Liaoning Province, China	0.2–1.0 ^c	60–64	Xu et al. (1995)
<i>B. yuanmingense</i>	9.5–16 h	China	1.0–2.0 ^c	62–64	Yao et al. (2002)
<i>Sinorhizobium (Ensifer) fredii</i>	3–4 h	China	2.0–4.0 ^a	60–64	Chen et al. (1988)
<i>B. huanghuaihaiense</i> sp. nov.	7–9 h	Huang-Huai-Hai, China	1.0 ^c	61.5	Zhang et al. (2012)
<i>B. daqingense</i> sp. nov.	10 h	Daqing city, Heilongjiang province, China	1.0 ^{***}	61.2	Wang et al. (2013)
<i>E. sojae</i>	3–4 h	Hebei province, China	1.0–3.0 ^a	60.9	Li et al. (2011b)
<i>E. xinjiangense</i>	3–4 h	Xinjiang Province, China	2.0–4.0 ^a	60.1–60.9	Chen et al. (1988)
<i>Mesorhizobium tianshanense</i>	Variable generation time	Xinjiang, China			Peng et al. (2002)
<i>R. tropici</i>	1.6–2 h	Brazil	1.0–2.0 ^b	59–63	Chen et al. (1995)
<i>Rhizobium</i> sp.	2–3 h	Brazil	2.0–4.0 ^d	59–65	Hungria et al. (2006)
			2.0–3.5 ^d	59–65	Hungria et al. (2006)

^a After 3–4 d of incubation
^b After 5–7 d of incubation
^c After 7–10 d of incubation
^d After 2–4 d of incubation

Sinorhizobium sp. III and *S. sojae*. Different levels of genetic differentiations were observed among these species. *S. sojae* was most divergent from the other test species and was characterized by its low intraspecies diversity and limited geographic distribution. There was no geographic isolation between *S. fredii* populations in different ecoregions in China (Guo et al. 2014).

5.3.2 *Slow Growing Isolates*

The slow growing root nodulating bacterium of soybean was initially termed *Rhizobium japonicum* which was later delineated into a separate genus *Bradyrhizobium* because of its slow growth. The slow growers able to form effective root nodules on soybean are distributed in four species of the *Bradyrhizobium* genus, namely, *Bradyrhizobium japonicum*, *Bradyrhizobium liaoningense*, *B. yuanmingense* and *Bradyrhizobium elkanii*.

Multilocus sequence analysis of the soybean rhizobia in the Asiatic Continent (Myanmar, India, Nepal, and Vietnam), revealed the presence of all the four *Bradyrhizobium* species viz., *B. japonicum*, *B. elkanii*, *B. liaoningense*, and *B. yuanmingense*. *B. japonicum* and *B. elkanii* were found to be dominant in the humid and temperate climates of the Northern hemisphere, *B. liaoningense* in the East and Southeast while *B. yuanmingense* from Northern to Southern hemisphere with climatic conditions ranging from humid equatorial or dry, hot and semiarid (Vinuesa et al. 2008; Appunu et al. 2008). Of particular interest is *B. yuanmingense*, a very promiscuous symbiont with very broad geographic and host ranges capable of nodulating besides soybean, *Lespedeza* sp. (Vinuesa et al. 2005a), lima beans (Ormeno-Orrillo et al. 2006), *Indigofera hirsute* (van Berkum et al. 2003) and *Vigna* species (Zhang et al. 2008). Vinuesa et al. (2005b) however suggest the presence of symbiotic ecotypes within this species as strain isolated from one host do not cross nodulate the other. In the different agro-eco-climatic soybean growing regions of India with conditions varying from hot, sub humid and neutral to slightly acidic soils to hot, semiarid and highly alkaline soils three major species viz., *B. japonicum*, *B. yuanmingense*, and *B. liaoningense* and rarely *Sinorhizobium* sp. were found to be the main symbiont of soybean (Annapurna et al. 2007; Appunu et al. 2008, 2009). Soils of the temperate region of Nepal were found to be dominated by *B. japonicum* while in the subtropical regions with acidic, moderately acidic, and slightly alkaline soils; *B. elkanii*, *B. yuanmingense*, and *B. liaoningense* were the dominant symbionts (Adhikari et al. 2012).

In the American Continent, *B. japonicum* and *B. elkanii* have been reported to be the dominant species nodulating soybean. Genetic diversity studies of soil samples taken from acidic to slightly alkaline soybean fields across nine different states in the United States revealed the prevalence of *B. japonicum* and *B. elkanii* (Shiro et al. 2013). Similar observations were also reported from Brazil (Giongo et al. 2008) and Paraguay (Chen et al. 2000). In Brazil, however, only *B. japonicum* and *B. elkanii* have been used as commercial inoculants to increase soybean yields. Since the Brazilian soils lacks indigenous soybean bradyrhizobia (Alberton et al.

2006), its entire naturalized bradyrhizobia population nodulating soybean possibly came with seeds and inoculants from United States.

In India, where soybean was introduced in 1960s, native rhizobial populations slowly grew and segregated. Initially the inoculant was a strain from USA, the slow growing *B. japonicum*. Over the years as soybean was cultivated in various parts of the country, with indigenous cultivars, soybean rhizobial population developed. Dominant group still seems to be slow growers *B. japonicum*, *B. liaoningense* and *B. yuanmingense* (SatyaPrakash and Annapurna 2006; Appunu et al. 2008), though fast growing soybean nodulating rhizobia have also been reported from the country.

Interestingly, slow growing bradyrhizobia nodulating soybean comprises a heterogeneous group (Menna et al. 2009; Delamuta et al. 2012, SatyaPrakash and Annapurna, 2006; Tian et al. 2012). Molecular characterization using genetic typing methods like rep-PCR, 16S-RFLP, *nifH* and *nodC*, and MLSA were carried out to analyze the genetic diversity of bradyrhizobia nodulating soybean by these workers. Several genospecies have been reported within the group (Appunu et al. 2008; Man et al. 2008; Han et al. 2009). This in part could be attributed to the existence of transfer of symbiotic genes within the group. Vertical transfer by and large is the main phenomena for transfer of symbiotic genes within the group (Man et al. 2008; Han et al. 2009). Lateral transfer of symbiotic genes have also been reported (Han et al. 2009) while horizontal transfer of symbiotic genes is a very rare occurrence within the group. Interestingly, Marchetti et al. (2010) observed that transfer of *nod-nif* genes from the rhizobium *Cupriavidus taiwanensis* enable the pathogenic *Ralstonia solanacearum* to infect and nodulate *Mimosa* sp. which evidently points to the importance of *nod-nif* genes and gene activation in the evolution of bacteria into rhizobia. Tian et al. (2012) further argued that in addition to vertical and lateral transfer of genes, rhizobia also recruits lineage specific genes through symbiotic interactions and environmental adaptations.

Among a few studies on soybean rhizobial diversity in Nepal, some genetically distinct *B. japonicum* strains compared to those in other Asian countries have been reported in a soil in the Kathmandu Valley (Vinuesa et al. 2008). Recently, the diversity of soybean bradyrhizobia was assessed in five mountain soils of Nepal ranging from 1500 to 2600 m in elevation with soil pH levels ranging from 5.2 to 6.2, and a dominant presence of *B. elkanii* with minor populations of *B. japonicum* and *B. yuanmingense* was reported (Risal et al. 2010).

Besides these group of well documented rhizobial symbionts, *Rhizobium tropici*, *Rhizobium* sp. (Hungria et al. 2006), *Rhizobium oryzae* (Peng et al. 2008), *Mesorhizobium tianshanense* (Chen et al. 1995) have also been reported from the root nodules of soybean.

5.3.3 Biogeography of the Root Nodule Symbionts

Differentiated rhizobial gene pools nodulating *G. max* have been reported in different ecoregions of China. Rhizobia nodulating soybean are known to exhibit biogeographic pattern with soil pH and temperature playing a dominant role in influencing

their genetic diversity and distribution (Man et al. 2008; Han et al. 2009; Zhang et al. 2011). Soil nutrients including available P, K and N also effect the distribution and diversity of soybean rhizobia. In the soybean growing soils of North China Plain, high N concentration favored *S. fredii*, *B. liaoningense*, and *B. yuanmingense* population while it had a negative effect on the *B. elkanii* (Zhang et al. 2011). Positive correlation between high content of available K and growth of *B. yuanmingense* was observed in Hebei Province of China while the same inhibited *B. japonicum*, *B. elkanii* (Li et al. 2011a, b). The authors also reported correlation between high available P in the soil and the distribution of rhizobia in the same region nodulating soybean. Broadly speaking, *Sinorhizobium* is dominant in alkaline-saline soil whereas *Bradyrhizobium* in neutral to acidic soil (Man et al. 2008; Vinuesa et al. 2008; Han et al. 2009; Li et al. 2011a, b; Zhang et al. 2011). In the subtropical and tropic regions of China with humid and acid soils soybean preferred *B. japonicum*, *B. elkanii* over *B. liaoningense*, and *B. yuanmingense* as symbionts (Yang et al. 2006; Man et al. 2008). In the humid climate of Northeast China having neutral to slightly acidic soils the dominant symbiont of soybean was *B. japonicum* but never *S. fredii* (Wang et al. 2009). Sawada et al. (1989) examined 85 Japanese indigenous soybean-nodulating rhizobial strains isolated from 46 soils around Japan for their hydrogenase uptake (Hup) trait and somatic serogroup identity and suggested a relationship between the distribution of serogroup and Hup phenotype strains. Minamisawa et al. (1999) examined 213 indigenous soybean bradyrhizobia isolated from six fields in Japan by fingerprint analysis with probes for RS α , RS β , *nifDK* and *hupSL*, and suggested that the diversity of bradyrhizobia in individual fields is associated with host plants and local soil conditions. Similar studies are lacking in India. No rhizobial map has been generated for soybean growing areas in the country. SatyaPrakash and Annapurna (2006) gave the first report on the genetic polymorphism existing in root nodulating bacteria from soybean grown in one field. Root nodule isolates from the four varieties were *B. japonicum* types, growing in 4–7 days with typical colonies which were found to be genetically distinct from the USDA and SEMIA strains of *B. japonicum* and *B. elkanii*. Appunu et al. (2008) showed that the diversity is wider than expected based on previous studies in various geographic areas and on the current taxonomy of soybean rhizobia. Notably, the diversity of the soybean symbionts appears to be conserved across the agricultural-ecological-climatic regions sampled. In recent times we have come across fast growing rhizobia able to nodulate Indian cultivars of soybean (unpublished).

How could bacteria of these two contrasting genera (*Bradyrhizobium* and *Sinorhizobium*) evolve into the microsymbionts of the same legume plant? Recent comparative genomics of soybean rhizobia revealed that the core genome of *Bradyrhizobium* is rich in lipid and secondary metabolism genes whereas the *Sinorhizobium* core genome had several gene clusters known to be involved in osmoprotection and adaptation to alkaline pH, corroborating the biogeographic pattern of distribution of soybean rhizobia (Tian et al. 2012) (Table 5.2). However, it would be very interesting if we could find out specific genes involved for recognition/nodulation in these two genera. For this we need to sequence a large number of genomes from each genus (*Bradyrhizobium* and *Sinorhizobium*) to reveal any distinct feature specific for each genus related to their symbiotic capacity and environmental adaptations

Table 5.2 Comparison of COG assignments between *Sinorhizobium* and *Bradyrhizobium* nodulating soybean. (Courtesy Tian et al. (2012). Some selected functional categories where distinct differences were noted. For more details please see the original reference)

Individual functional categories	Genus specific genes	
	<i>Sinorhizobium</i>	<i>Bradyrhizobium</i>
J: Translation, ribosomal structure and biogenesis	8	18
L: Replication, recombination and repair	9	16
V: Defense mechanisms	6	11
N: Cell motility	16	25
F: Nucleotide transport and metabolism	16	8
E: Amino acid transport and metabolism	75	97
I: Lipid transport and metabolism	16	54
C: Energy production and conversion	39	55
Q: Secondary metabolites biosynthesis, transport and catabolism	10	45
R: General function prediction only	75	113

5.4 Conclusion

Glycine max (soybean) is one of the most important legume crops in the world thought to have originated in East Asia. Rhizobia that nodulates soybean represent a heterogeneous group. At least five rhizobial species have been reported as its microsymbionts by independent studies. In India, where soybean is cultivated in more than 10 million ha a large native population has developed which needs to be studied for its genetic diversity. Earlier reports by researchers have indicated at the heterogeneity existing among the strains forming root nodules on different cultivars. As the crop is grown in different agro-climatic regions in the country, it would be interesting to bio-prospect these strains. Recent comparative genomic studies have revealed that there is no gene specifically shared by *Sinorhizobium* and *Bradyrhizobium* to establish symbiosis with soybean. However, a large genetic diversity exists in the symbionts nodulating soybean. Recombination seems to have contributed to this diversity of the core genome of these symbionts.

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Chapter 6

Diversity and Function of Bacterial Assemblages in Savanna Vegetation Soils

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Abstract Savannas can be found in North and South America, Africa, Asia, and Oceania. The Cerrado is a vast savanna located mainly in the central region of Brazil. Although, Cerrado ecosystems are similar in vegetation structure, differences in soil characteristics influence the microbiota. Throughout the world savannas are rapidly being converted to agricultural and urban uses, altering physical and chemical properties of the soil, as well as microbial diversity through changes in bacterial and fungal richness, community structure, and activity. The studies addressed in this review describe soil microbial communities present in Cerrado ecosystems, which are dominated by Acidobacteria, Proteobacteria, and Actinobacteria. We highlight the importance of microbial communities to ecosystem services such as nutrient cycling, regulation of biogeochemical processes, and contribution to net primary production. Sustainable development based on the use of natural resources requires a better understanding of the microbial processes and genetic resources in this biome.

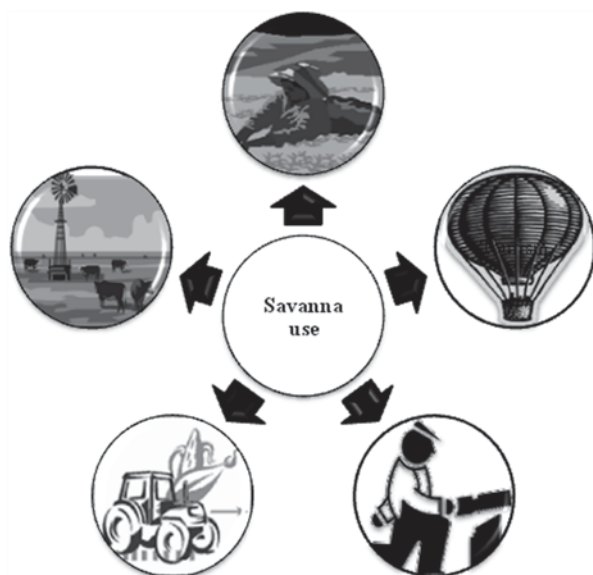
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Fig. 6.1 Principal forms of land use in the Cerrado



6.1 Introduction

A typical savanna consists of a continuous herbaceous layer and a discontinuous stratum of shrubs and trees, although the relative representation of these life forms varies considerably across savanna types (Frost et al. 1986; Scholes and Archer 1997). Savannas cover 12% of the global land surface and include arid shrublands, lightly wooded grasslands, deciduous woodlands, and dry forest (Scholes and Archer 1997; Sankaran et al. 2004). Savannas can be found in South and North America, Africa, Asia, and Oceania, where they support a large proportion of the world's human population and most of its rangeland and livestock (Scholes and Archer 1997). Savanna ecosystems are known by different names in each continent and have different physiogenomic characteristics (Table 6.1). Their great diversity allows for a variety of land uses (Fig. 6.1), but the increasing exploitation of these ecosystems has highlighted the need for a comprehensive conservation strategy.

Although studies differ regarding the relative importance of specific ecological determinants of savanna structure and function, the primary factors are water, nutrient availability, fire, herbivory, and human intervention. The first two are considered primary controls of the ecosystem, and the others are considered modifiers of the environment (Goedert et al. 2008).

Savanna soils are characterized by low levels of nutrients and exchangeable minerals (calcium, magnesium and phosphorus) and low cation-exchange capacity. In addition, these highly weathered soils contain large amounts of exchangeable aluminum, which can reach toxic levels (Frost et al. 1986). The release of soil nutrients is directly influenced by the availability of water in the soil, which is determined by rainfall (Medina and Silva 1990). Water is also a key determinant for the composition and structure of soil microbial communities. Soil microorganisms

Table 6.1 Distribution and characteristics of savannas

Continent	Local name	Main countries	Physiognomic characteristics	Reference
North America	Savannas	United States	Pinion juniper forest, oak and ponderosa pine savanna	McPherson (1997)
	Savannas	Mexico	Pinion juniper forest	McPherson (1997)
South America	Cerrado	Brazil	From grasslands to forest formations	Ribeiro and Walter (1998)
	Llanos, Savannas	Colombia and Venezuela	From grasslands to forest formations	Romero-Ruiz et al. (2010)
West Africa	Savannas	Senegal, Guinea, Côte D'Ivoire, Mali, Ghana, Benin, Nigeria, Cameroon, and Chad	Woodlands	Mistry (2000)
Central and South Africa	Savannas	Angola, Democratic Republic of Congo, Zambia, Malawi, Zimbabwe, Mozambique, Namibia, Botswana, and South Africa	Woodlands	Mistry (2000)
East Africa	Miombo	Ethiopia, Somalia, Uganda, Kenya, and Tanzania	Woodlands	Mistry (2000)
Asia	Savannas	India, Burma, Laos, Thailand, Vietnam, and Cambodia	Grass associated with thorny bushes	Mistry (2000)
Oceania	Savannas	Australia	Trees, shrubs, and grasses	Mistry (2000)

are important drivers of plant diversity and productivity, with direct effects via root-associated organisms that form mutualistic or pathogenic relationships with plants, and indirect effects via free-living microbes that alter rates of nutrient supply and the partitioning of resources (van der Heijden et al. 2008).

Savanna ecosystems are under intense pressure due to conversion to agricultural and urban use, resulting in loss of above- and below-ground biodiversity. Because of the high level of endemism and rapid loss of habitats, the Cerrado is considered a biodiversity hotspot (Myers et al. 2000). Here, we present a review of studies on soil bacterial communities (diversity and activity) in native savannas ecosystems and

agroecosystems, focusing on the Brazilian Cerrado. We will first describe bacterial communities associated with different savanna vegetation types and soils, and then discuss the effects of fire and land use changes.

6.2 Soil Bacterial Communities: Effects of Vegetation Types and Fire

The Cerrado is the largest neotropical savanna formation in America (Eiten 1972), comprising 24% of the total land area of Brazil. Located in Central Brazil, this biome shares transition areas with adjoining biomes such as the Amazon rainforest, the Atlantic Forest, the Pantanal (wetlands), and the Caatinga (semi-arid shrublands). Thus, the Cerrado plays an important role as a wildlife corridor between other biomes.

The Cerrado has a tropical semi-humid climate. Its annual mean temperature ranges from 26°C (North Brazil) to 20°C (South Brazil), and the annual mean rainfall ranges from 1200 to 2000 mm, mainly concentrated in November through March. Landscape fires are common in natural Cerrado areas (Furley 1999), but fire is also used by humans to clear areas for cultivation and eliminate undesirable species.

Cerrado is characterized by high biodiversity in a mosaic of vegetation types that vary according to the density of woody plants, ranging from forest formations to savannas and grasslands (Ribeiro and Walter 1998). Oxisols (US Soil Taxonomy) are the predominant soil type, comprising 46% of Cerrado soils, whereas Entisols and Ultisols/Alfisols represent 15.2% and 8.2%, respectively (Reatto et al. 1998). Accordingly, Oxisols are the soils most often described in soil microbial diversity studies carried out in the Cerrado. These soils are acidic, nutrient-poor, highly weathered, well-drained, have low levels of available phosphorus, and are rich in iron and aluminum oxides. Generally, they are red or yellow with a fine texture and high water conductivity (Furley 1999).

Soil bacterial communities in the Cerrado differ according to the vegetation (grasslands, shrublands, and forest formations) (Araujo et al. 2012). Overall, the most abundant microbial phyla in Cerrado soils are Acidobacteria, Proteobacteria, and Actinobacteria (Fig. 6.2, Table 6.2) (Araujo et al. 2012), with Acidobacteria accounting for approximately 50% of the 146 sequences in a 16S rRNA gene library constructed from DNA isolated from Cerrado soil (de Castro et al. 2011). A study of Cerrado native areas (savanna, grassland, or forest soils) reported that Acidobacteria subgroup Gp1 was the most abundant group, suggesting that it is well adapted to dystrophic soils, changes in water soil content, and low pH (4.1–4.9) (Araujo et al. 2012).

Acidobacteria are slow-growing bacteria and most abundant in environments with low nutrient levels, as evidenced by the negative correlation between Acidobacteria abundance and carbon availability in the soil (Fierer et al. 2007; Nemergut et al. 2010). Acidobacteria have metabolic abilities related to carbon degradation and are tolerant to water stress, which may account for their ability to thrive under

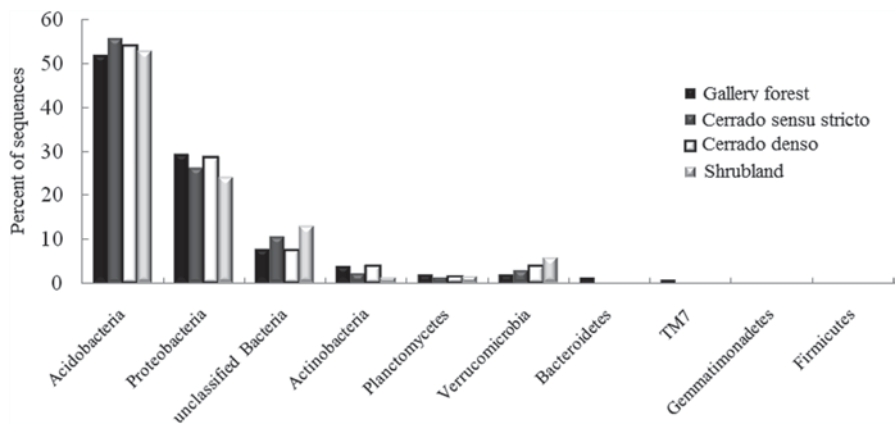


Fig. 6.2 Relative distribution of bacterial phyla present in different Cerrado phytophysiogenomies as determined by 16S rDNA sequences analysis

Table 6.2 Predominant phyla found in the Brazilian savanna soils and the methods used to describe them

Area	Method	Predominant Phyla	Reference
Cerrado (Federal District, Brazil)	16S clone library (ABI Prism 377)	α -Proteobacteria Acidobacteria Actinobacteria	Quirino et al. (2009)
Cerrado (Federal District, Brazil)	RISA, T-RFLP 454 pyrosequencing	Acidobacteria Proteobacteria	Araujo et al. (2012)
Savanna (Etosha National Park, Namibia)	16S PhyloChip G2	Proteobacteria Firmicutes Actinobacteria	Ganz et al. (2012)
Cerrado (Goiás, Brazil)	16S 454 pyrosequencing	Acidobacteria Proteobacteria Actinobacteria	Rachid et al. (2013)
Cerrado (Minas Gerais, Brazil)	16S 454 pyrosequencing	Proteobacteria Acidobacteria Actinobacteria	Rampelotto et al. (2013)

RISA ribosomal intergenic spacer analysis, *T-RFLP* terminal restriction fragment length polymorphism

adverse conditions such as nutrient-limited, acidic soils that contain high aluminum levels and are subject to seasonal fluctuations in water availability, as occurs in the Cerrado.

Within the vegetation gradient in the Cerrado, which ranges from grasslands to forestlands, the gallery forest has the highest biomass and activity (Mendes et al. 2012). Rarefaction analysis shows that the gallery forest also exhibits a greater bacterial richness (Araujo et al. 2012). Compared with other Cerrado habitats, this forest formation has a higher density of tree species, higher levels of organic carbon and available phosphorus, and lower water stress during the dry season (Araujo

et al. 2012). In addition, the gallery forest exhibits a higher diversity of decomposer species from the orders Xanthomonadales and Legionellales (γ -Proteobacteria), and Burkholderiales (β -Proteobacteria) (Araujo et al. 2012).

A recent study reported that precipitation was second only to vegetation type as a determinant of soil biological function in Cerrado soils (Mendes 2012). The beginning of the wet season is associated with an increase in microbial biomass (Nardoto and Bustamante 2003; da Silva 2004), microbial activity, organic carbon, and nitrification (da Silva 2004). These changes in soil water content appear to mask the effects of other factors, such as fire, on microbial communities (da Silva 2004; Viana et al. 2011). In a study investigating the effects of the first rain events on dry Cerrado soil, denaturing gradient gel electrophoresis (DGGE) analysis and measurement of CO₂ and NO fluxes revealed that the bacterial community structure responds rapidly after artificial water addition (Pinto et al. 2006). Even in a gallery forest, where soil water stress is less intense, bacterial ribosomal intergenic spacer analysis (RISA) has shown that soil bacterial communities differ between the wet and dry seasons (Lira 2012). Soil samples collected in different positions along a topographic gradient and before and after an accidental fire also differed in community structure. Several bands in the RISA gel could be visualized under all conditions, indicating the resilience of some species to these environmental impacts. Sequencing of the most predominant bands revealed that most of these species belonged to the Actinobacteria and Proteobacteria phyla; however, Acidobacteria and Gemmatimonadetes were also present in smaller numbers (Lira 2012).

Unlike most savanna formations, gallery forests are not fire-prone ecosystems. Fires in Cerrado savanna formations are fueled primarily by the herbaceous stratum (<6 mm) (Miranda et al. 1996) and progress rapidly. Although the temperature above ground can reach 85 to 840 °C (Miranda et al. 1993), the temperature below ground changes little, with a maximum of 55 °C at a depth of 1 cm (Castro-Neves and Miranda 1996). This soil temperature is not lethal to microorganisms (Wells et al. 1979); however, long-term effects of fire can include changes in vegetation cover and altered soil characteristics such as pH and nutrient availability. da Silva (2004) and Viana et al. (2011) evaluated the effects of prescribed burning on soil microbial communities in two vegetation types of native Cerrado (woodland cerrado and shrubland). Although fire influenced soil moisture and pH, no significant differences were observed between microbial communities in the burned and unburned areas of either vegetation type. The authors concluded that seasonal changes exert a greater influence than fire on soil microbial communities, as assessed by DGGE (da Silva 2004) and PLFA (Viana et al. 2011) analyses, with an increase of microbial biomass carbon apparent soon after the first rains.

Response to nutrient deposition has been evaluated in soil bacterial communities of native Cerrado ecosystems by barcoded pyrosequencing of bacterial 16S rRNA genes (da Silva 2012). Changes in the bacterial community in response to the addition of calcium, nitrogen, phosphorus, or the combination of nitrogen and phosphorus were associated with the presence/absence of certain groups and to changes in abundance of common groups.

The cultivation of alien grasses affects soil microbes and represents a serious threat to Cerrado biodiversity (Pivello 2011). African grasses were introduced in the region to improve pasture productivity, but their invasion of conservation units is now widespread (Pivello et al. 1999a, b). DGGE analyses of soil samples collected under a native grass (*Echinolea inflexa*) and invasive African grass (*Melinis minutiflora*) have demonstrated a clear differentiation of the bacterial communities (da Silva 2012).

6.3 Land use Changes and Bacterial Response in Savanna Soils

Savannas all over the world are undergoing profound transformations, and the Cerrado is no exception. For instance, land use transformation has reached approximately 48 % (989,817 km²) in the Brazilian Cerrado (CRS/IBAMA 2011) and approximately 13 % (2,228 km²) alone in the savannas of the Colombian Llanos Orientales (Romero-Ruiz et al. 2012). Deforestation in Central Brazil began in the 1960s with the establishment of the Brazilian Federal District. By the 1970s, the Cerrado biome was occupied by pastures, and later by crops. An estimated 50 % of the Cerrado is considered potentially arable land, able to be incorporated into agriculture, pasture for livestock, or forestry production (Lopes 1996). These land use changes result in fragmentation of the biome. This is particularly true when it is converted to cropland, because of the need to utilize the most favorable slope and soil conditions (Carvalho et al. 2009). The Cerrado biome is currently a leader in grain production and one of the largest of the world's agricultural frontiers. More recently, sugarcane production and the increasing demand for biofuels are contributing to ecosystem transformation (Sano et al. 2010).

Firstly, we will present the compilation of the available information on soil properties and how they respond to changes in soil cover and use, and then we present the data regarding the soil bacterial communities under different land uses in savannas.

6.3.1 Land use Changes and Soil Properties

We reviewed a set of physicochemical parameters from the surface soil layer (0- to 10-cm) collected from native, agricultural, and pasture sites; these parameters included soil texture (content of sand, clay, and silt); pH; concentrations of phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sodium (Na), and aluminum (Al); potential acidity (H+Al); cation-exchanged capacity (CEC); organic carbon (OC); microbial biomass (MB); and total nitrogen (TN). The physicochemical parameters chosen for evaluation varied among the included studies. For example, Frazão et al. (2010) analyzed nitrogen concentration, microbial biomass, and microbial activity in sandy soils of the Brazilian Cerrado under different land use

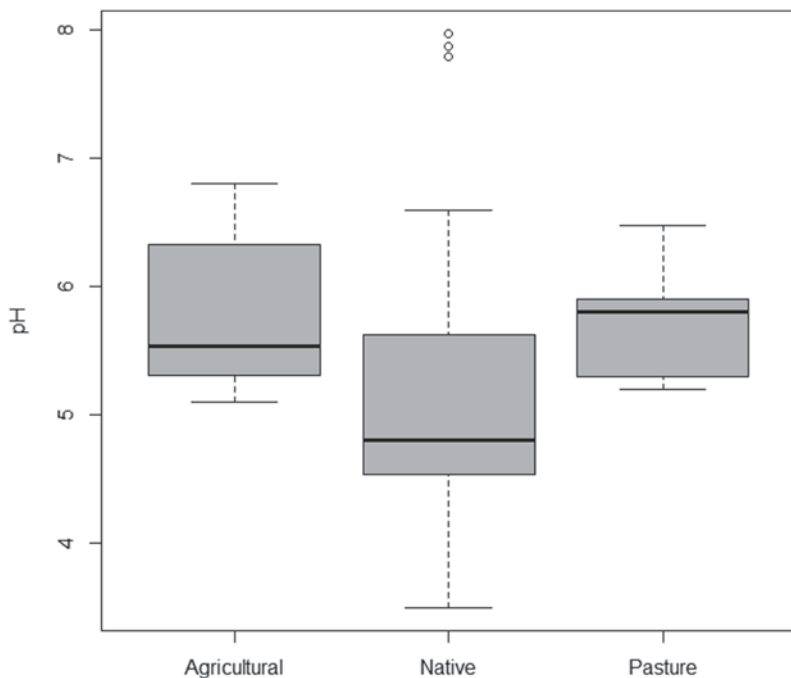


Fig. 6.3 Boxplot of pH measurements of three types of savanna soil (native, agricultural, and pasture) using R software v.3.0.2. (R Core team 2013)

conditions (pasture and croplands) and in different seasons (dry and wet). These parameters were considered relevant to evaluate the role of microbial attributes in the conversion of native Cerrado to pasture and agricultural systems. The researchers concluded that microbial components of the soil were more strongly influenced by seasonal conditions than by soil management systems. However, other parameters such as percentage of sand and silt, percentage of organic carbon, or amount of phosphorus and potassium were not described.

Comparisons are possible only for factors that were evaluated in most studies, such as pH. Soil pH directly influences nutrient availability for plants and microbes, and is one of several factors affecting bacterial diversity (Tripathi et al. 2012). In the included studies, the median pH value was 5.20 for all sites analyzed, with median pH 4.80 for native savanna areas, and median pH 5.60 for modified sites (pasture or farm) (Table 6.3). The native savanna soils generally had lower pH values than modified sites (Fig. 6.3), with the exception of African soils, which had higher pH (outliers), as well as Ca, Mg, and K levels.

Another parameter usually described in these studies is soil texture. The relative amounts of sand, clay, and silt influence soil moisture through their effects on water retention capacity, which affects microbial community composition (Nielsen et al. 2010). Although several papers did not describe these parameters, where reported these values were similar across the analyzed sites (protected or modified), indicating a predominance of clayey soils (Table 6.3).

Table 6.3 Physicochemical parameters of three types of savanna soils (native, Pasture, and Agricultural) from several papers published 2006–2013. (Data represent the median values of the included studies (Pinto et al. 2006; Carvalho et al. 2007; Quirino et al. 2009; Bresolin et al. 2010; Frazão et al. 2010; Peixoto et al. 2010; Viana et al. 2011; Araujo et al. 2012; Ganz et al. 2012; Mendes et al. 2012; Vinhal-Freitas et al. 2013; Rachid et al. 2013; Rampelotto et al. 2013; Rossi et al. 2013; Souza et al. 2013))

Soil type	Sand (%)	Silt (%)	Clay (%)	pH (H ₂ O)	P (mg dm ⁻³)	K (mg dm ⁻³)	Ca (cmol _c dm ⁻³)
Native	18.00 (15) ^a	12.50 (15)	68.00 (15)	4.80 (23)	1.80 (18)	37.00 (19)	0.25 (16)
Pasture	23.40 (7)	18.50 (7)	62.20 (7)	5.80 (8)	1.35 (2)	45.00 (3)	0.17 (3)
Agricultural	20.80 (6)	8.20 (7)	71.00 (9)	5.54 (16)	14.80 (13)	75.60 (16)	3.14 (16)

Soil type	Mg (cmol _c dm ⁻³)	Al (cmol _c dm ⁻³)	H + Al (cmol _c dm ⁻³)	CEC (cmol _c dm ⁻³)	OC (%)	MB (mg C kg ⁻¹)	TN (%)
Native	0.10 (15)	1.00 (9)	8.40 (15)	10.11 (13)	2.75 (22)	557.00 (7)	0.16 (10)
Pasture	0.13 (3)	0.21 (2)	3.43 (2)	2.79 (1)	2.31 (8)	510.00 (4)	0.19 (3)
Agricultural	1.18 (14)	0.00 (11)	3.80 (16)	8.39 (14)	2.53 (13)	231.76 (4)	0.17 (8)

P phosphorus, *K* potassium, *Ca* calcium, *Mg* magnesium, *Na* sodium, *Al* aluminum, *H + Al* potential acidity, *CEC* cation-exchanged capacity, *OC* organic carbon, *MB* microbial biomass, *TN* total nitrogen

^a Numbers in parenthesis represent the number of studies used to calculate the median value

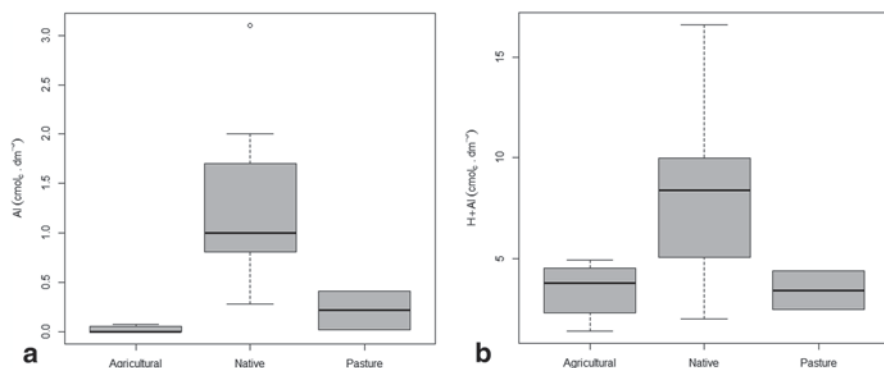


Fig. 6.4 Boxplot of **a** concentration of aluminum (Al), and **b** potential acidity (H+Al) from three types of savanna soil (native, agricultural and pasture) performed using R software v.3.0.2. (R Core team 2013)

In our study we noted high values for Al and H+Al in several native sites analyzed, whereas modified sites showed relatively low values (Fig. 6.4). This outcome was expected because liming is used to increase pH in agricultural lands. Wherein it was recorded near 7.0, increase soil Ca, Mg, and K concentrations, as noted in the agricultural sites (Table 6.3). The high Al and H+Al levels in Cerrado soils prevent the adsorption of nutrients—with the exception of phosphorus, which is more adsorbed and less available—making these soils inappropriate for agriculture without pH correction.

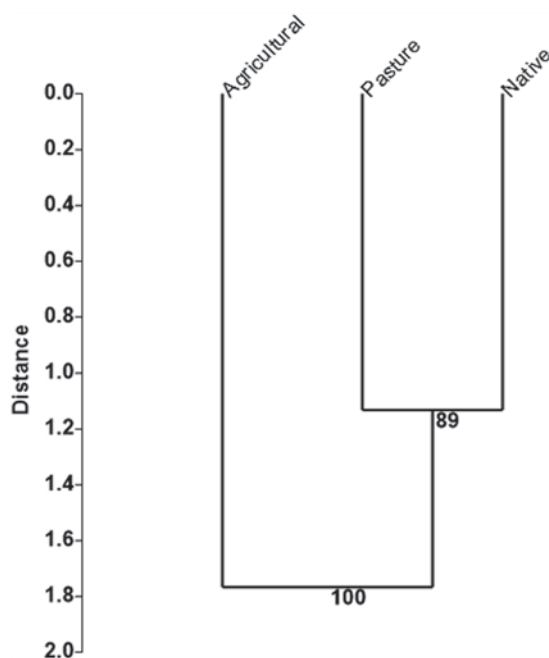
Other nutrients such as N and K are commonly added to agricultural fields. These important plant nutrients are generally less available in native soils, and changes in N and K alter bacterial community composition (Horner-Devine et al. 2004). Agricultural sites were ten times more likely to show high P concentrations (Table 6.3), whereas, TN concentrations were similar among the studied sites. However, to compare sites it is recommended to evaluate available forms of nitrogen (ammonium, nitrate, or nitrite). In addition, the studies showed that the concentration of organic carbon (OC), derived from decomposition of dead plants, animals, and other organisms, was similar between converted sites and native savanna ecosystems.

Thus, in these studies native savanna sites showed high concentrations of Al and H+Al, and low pH values, whereas agricultural sites showed high concentrations of Ca, Mg and K. Pasture sites showed a pattern similar to that of native sites (Fig. 6.5), differing mainly in silt content, microbial biomass and total nitrogen

6.3.2 Land use Changes and Soil Bacterial Communities

To determine microbial indicators of soil quality in savannas, several studies evaluated the impact of land use change on soil biochemistry and microbial diversity, reporting a reduction in bacterial and fungal richness (de Castro et al. 2008; Quirino

Fig. 6.5 Cluster analysis of savanna soils (native, agricultural, and pasture) on the basis of physicochemical parameters using PAST software v.2.17 (Hammer et al. 2001). Single linkage algorithm associated Euclidean similarity measure was used (correlation coefficient: 0.9667; bootstrap: 10,000)



et al. 2009) and modification of the microbial community structure (Pinto et al. 2006; Roesch et al. 2007; Bresolin et al. 2010; Viana et al. 2011; Rampelotto et al. 2013; Souza et al. 2013). Most of the studies (published from 2006 to 2013) assessed microbial biomass or used molecular fingerprinting techniques to understand these changes, but some studies characterized soil bacterial assemblages at the taxon level using high-throughput 16S rRNA gene sequencing. For example, an area of the Brazilian Cerrado under soybean monoculture showed 17–66% (depending on the crop stage) less microbial biomass carbon than a native area (Bresolin et al. 2010). The study pointed out that even using no-tillage methods, significant differences were observed in microbial biomass, community structure, and composition after 30 years of continuous cultivation. This study also reported that microbial communities respond to specific stages of crop development. These findings emphasize the importance of the quality of vegetation cover and activity (e.g., root exudates) for soil microbial dynamics.

Rampelotto et al. (2013) reported that Acidobacteria and Proteobacteria each comprised approximately 30% of the 126,957 high-quality bacterial 16S rRNA gene sequences obtained from Cerrado soils under different land uses. They assessed the conversion of natural forest to sugarcane field (fertilized) and coniferous forest by the pyrosequencing of 16S rRNA genes. Land use change increased the abundance of Gp2 (Acidobacteria) and *Acinetobacter* (γ -Proteobacteria) in the coniferous forest, and Gp6 (Acidobacteria) in the sugarcane field. The number of bacterial groups and their abundance in the sugarcane field were higher than in the natural forest, possibly because of fertilization. Moreover, some groups, such as Gp7

(Acidobacteria), *Blastococcus* (Actinobacteria), *Solirubrobacter* (Actinobacteria), and *Anaerolineaceae* (Chloroflexi) were found exclusively in the sugarcane field. These data revealed that land use change does not always reduce bacterial diversity; it also can lead to structural changes with regard to the presence of specific groups.

Rachid et al. (2013) evaluated the impact of two sugarcane cultivation systems on bacterial assemblies in Cerrado soils by pyrosequencing and qPCR of the 16S rRNA gene. They found that the number of operational taxonomic units did not differ among the following three soil types: (1) burnt sugarcane field (uncovered soil after harvest), (2) green sugarcane field (soil covered with the vegetal residues after harvest), and (3) native Cerrado soil. However, copy numbers of the 16S rRNA gene were significantly lower in the agricultural soils, and members of the Firmicutes phyla varied in abundance among the soil types. In addition, fewer Acidobacteria classes were detected in the cultivated sites, especially in the burnt sugarcane field, although this difference was not significant. On the other hand, the average relative frequency of Verrucomicrobia was higher in the burnt sugarcane field. Contradicting the idea that all bacteria exist everywhere, this study revealed that only a small fraction of operational taxonomic units was present in all samples. Although these findings demonstrate that sugarcane cultivation transforms the soil microbial community structure, microbial communities in soils under green sugarcane management were more similar to those of soils under native vegetation, indicating the lower impact of green sugarcane management on bacterial assemblies. This is a good example of how knowledge of soil bacterial communities can guide land management to maintain ecosystem balance.

The type of agriculture soil treatment used also influences the level of change in the microbial community. Peixoto et al. (2006) reported that soils under no-tillage treatment are more similar to forest soils than to soils under conventional tillage. Furthermore, the predominant species in soils under conventional tillage appear to be plant pathogens (Souza et al. 2013). Functionally, phosphatase activity is lower in soils under conventional tillage than in no-tillage or native Cerrado soils (Peixoto et al. 2010). Compared with other soil management methods, no-tillage management improves soil quality and promotes microbial content and activity (Peixoto et al. 2010; Vinhal-Freitas 2013).

Results of phospholipid fatty acid analysis of native Cerrado soils converted to pasture soils (Viana et al. 2011) reinforce previous studies reporting that vegetation cover and land conversion are important factors affecting microbial assemblies in savannas. However, these shifts in the microbial community are due to differences in the relative abundance of the same groups. Structurally, Gram-negative bacteria are more abundant in pasture soils than in native soils, which may be due to the abundance of Proteobacteria (Quirino et al. 2009; Rampelotto et al. 2013). However, Proteobacteria are abundant in many soils, which indicates that although abundance of the phylum as a whole may not be affected by land use change, subgroups within the phylum may be affected (Rampelotto et al. 2013).

Biogeographic distributions of bacteria in terrestrial biomes are also affected by soil pH (Lauber et al. 2009). In Cerrado pastures, high pH correlates with a greater abundance of Gram-negative bacteria such as *Azospirillum* (a nitrogen-fixing organ-

ism) (Viana 2002). Nonetheless, the transition from wet to dry seasonal conditions in the Cerrado biome was the factor most often implicated in changes in soil microbial diversity. Seasonal variation in water content leads to changes in soil pH and microbial biomass carbon, which affects the composition of bacterial populations, as assessed by polymerase chain reaction (PCR)-DGGE (Bresolin et al. 2010). In Cerrado soils P availability is a critical limiting factor, and the P contained in microbial biomass is more readily available through mineralization than total organic P, making it an important source for plant nutrition (Resende 2001). In this sense da Silva (2004) suggested that adding P to the soil would promote structural differentiation of bacterial communities. This idea was later confirmed by DGGE analysis, which showed that nutrient addition, specifically P, produced a greater difference in the soil microbial community than the time of conversion of Cerrado to pasture, which resulted in another group of the DGGE cladogram of non-fertilized pasture and native shrubland. In a study evaluating microorganism response to P addition in Cerrado soil, Ferreira et al. (2013) detected a relationship between P content and microbial activity and respiration in no-tillage and conventional-tillage systems.

The studies cited above demonstrate specific ways that soil microbes respond to land use changes in the savanna. However, the complexity of interactions in the soil environment makes it difficult to predict how disturbances affect individual processes and the ecosystem as a whole (Andr  n et al. 2008). Another problem faced by microbial ecologists is the use of ecological approaches more suitable for analyzing macroorganisms, which do not take genetic microbial dynamics into account (Martiny et al. 2006; Bapteste and Dupr   2013). In addition, two properties intrinsic to microbe communities make their behavior unpredictable after a disturbance: resilience (ability to recover rapidly) and functional redundancy (ability of multiple taxa to perform the same function) (Allison and Martiny 2008). Interactions between diverse components of an ecosystem hamper the modeling of effects arising from disturbances (Jeltsch et al. 2010), and the interactions of microorganisms are even less intelligible, primarily because of their dynamics. Therefore, the ability to predict microbial responses to perturbation in time to manage potential damage requires additional data.

6.4 Microorganisms in Ecosystem Function: Taxa vs. Function

Soil microorganisms are key players in ecosystem functions through the decomposition of plant residue and organic matter into stable organic matter, which serves as a reservoir of nitrogen, phosphorus, sulfur, metal elements, and other nutrients (Horwarth 2002). Microbes act as source and sink of important nutrients, maintaining the global carbon budget, and forming biominerals such as carbonates, phosphates, oxalates, silicates, sulfates, sulfides, oxides, and hydroxides. Moreover, they are responsible for biogeochemical processes in the development of soil and maintenance of its properties. In summary, microbes regulate a large proportion of the

net primary production in ecosystems. In savanna soils, Proteobacteria are known to play important roles in carbon, nitrogen, and sulfur cycling; therefore, specific taxa of this phylum should be analyzed to better understand the impact of land use change (Rampelotto et al. 2013). For example, α -Proteobacteria is most abundant class of Proteobacteria in native areas (summarized in Table 6.2). This may be due to the abundance of nitrogen-fixing organisms, such as the genus *Bradyrhizobium* in native savanna Cerrado and forests and other families within the order Rhizobiales (Araujo et al. 2012). These microorganisms may be important in N-limited soils, such as those found in the Cerrado (Nardoto and Bustamante 2003).

On the other hand, actinobacteria are more abundant in pasture soils than in native Cerrado soils. This phylum of bacteria is known for the production of antibiotics and other secondary metabolites (Petinate et al. 1997; Gomes et al. 2000), suggesting that they have established themselves in pasture soils by using these chemicals to outcompete other bacteria. Actinobacteria also contain genes involved in desiccation and resistance to ultraviolet light (LeBlanc et al. 2008). In Cerrado soils, the predominance of actinobacteria may be related to its resistance to aluminum, as this soil has high aluminum content (Araujo et al. 2012). In addition, actinobacteria appear to be resistant to heavy metals (Gremion et al. 2003). The Cerrado has been suggested as a potential area for the isolation of new actinobacteria species (da Silva 2012). da Silva (2012) described 2152 complete or partial isolates of actinobacteria from Cerrado soil samples that differed phenotypically from previously described members of this phylum. Some actinobacteria isolated from Cerrado soil samples exhibit chitinolytic and proteinase activities (Petinate et al. 1997; Gomes et al. 2000; Nascimento et al. 2005). Other studies have also described the isolation of novel actinomycetes and acidobacterial species from Cerrado soil (Albuquerque de Barros et al. 2003; de Castro 2012).

Despite the importance of soil microbial communities, microbial ecologists know little about their distribution patterns. Understanding forces that drive this distribution can clarify the factors—contemporary or historical—that influence the community structure (Martiny et al. 2006). Martiny et al. (2006) found that taxonomically, microbial distribution displayed patterns such as species-area relationship and isolation by distance, supporting the hypothesis of microbial biogeography. If this supposition is correct, then some microorganisms may be restricted to a specific biome, highlighting the importance of considering microbes along with their habitats when discussing global sustainability.

Microbial taxa respond differently to diverse disturbances, decreasing or increasing in abundance. However, these oscillations in abundance reveal little about the behavior of the ecosystem after a given event. The functionality of the microbial community can better answer this question, because functionally redundant taxa exist within a community (Allison and Martiny 2008). However, microbial ecologists debate the relative importance of microbial taxa in relation to community function (Martiny et al. 2006; Green et al. 2008). Recently, Martiny et al. (2013) analyzed the distribution of 89 functional traits of bacteria and archaea using 2229 prokaryotic genomes (26 phyla) and the utilization patterns of 70 organic carbon substrates of 738 strains (5 phyla). The results demonstrated that bacteria transfer most traits

(93 %) vertically, and the more complex the pathway, the narrower its distribution phylogenetically. This finding reveals a closer relationship between taxa and function, and emphasizes the relevance of diversity for the function of a microbial community. Consequently, we should consider both microbial taxa and function. Additionally, the functional stability of a system depends on the microbial community composition (who is there) rather than abundance (how many are there) (Griffiths et al. 2004). Thus knowing who is where, why they are there, and what they are doing may help us understand how disturbances like climate and land use changes influence microbial assemblages and, consequently, ecosystem function (Green et al. 2008).

6.5 Conclusion

Despite its biological and ecological relevance, only about 2.2% of the Cerrado is protected in conservation units. Researchers are investigating this biome as a carbon sink or important area to target for conservation of Brazil's soil and water (Marris 2005). Currently, only two kingdoms of life are considered with regard to conservation—plants and animals—even though the survival of many macroorganisms depends on microbial activities. The conservation of organisms and their habitats is a complex issue that raises an important question: why protect an endangered population? This issue becomes more complicated if we do not know exactly how we will be affected by the disappearance of a species or changes in its behavior. That is the case for microorganisms.

Microbes carry out many essential services and functions (Giambelluca et al. 2009). They provide regulation services such as biogeochemical processes, thereby influencing ecosystem function and formation (Bell et al. 2005; Ducklow 2008). In addition, their metabolic products are used in pharmaceuticals, food, fuels, pollutant removal, and industrial processes. To date, there are no published studies describing microbial services in savanna ecosystems. For sustainable development in this biome (and others), it is imperative to achieve a better understanding of the microbial processes governing these services and to preserve the genetic resources of soil microorganisms. This will guarantee the provision of microbial services.

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Chapter 7

Diversity of Plant-Growth-Promoting Rhizobacteria Associated with Maize (*Zea mays* L.)

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Abstract Plants and most of the microorganisms in the rhizosphere have important interactions. Many microbial species inhabiting rhizosphere are known to exert beneficial effects upon *Zea mays* (maize) growth, and these are generally referred to as plant growth promoting rhizobacteria (PGPR). The ecological role of these microorganisms in soil is essential, since they participate in the biogeochemical cycles of the main nutrient elements in the ecosystems. There are many microbial communities that may benefit the maize plants. Since decreasing the use of fertilizers in agriculture is a worldwide necessity, especially on the maize crops, there is a great interest in understanding bacterial diversity to better explore their potential. Soil factors, plant type, root exudates and agricultural management are the factors that determine the community composition within the rhizosphere. Applications of molecular methods to access soil microbial diversity are reviewed.

7.1 Introduction

Maize (*Zea mays* L.) is one of the oldest crops and is cultivated in many areas of the world. This grain is used in human food and in animal feed and for generating raw industrial materials. It is one of the most widespread crops in the world, concerning both production and consumption. In the period 2010/2011 world production was around 821 million t and, of these, about 38.53 % were produced by United States of America, 21.08 % by China and 6.7 % by Brazil (USDA 2012). Thus, in the global scenario, Brazil stands out as the third largest producer, with about 54.37 million t of grain produced in an area of approximately 12.93 million ha (CONAB 2012).

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Despite the position of Brazil in world scenario and although Brazilian productivity has grown systematically over the last years, from about 2200 kg ha⁻¹ in 2000 to about 5000 kg ha⁻¹ in 2011 (CONAB 2012), the average productivity is still low.

The growth and development of maize plants is limited by biotic and abiotic factors (Kadioglu et al. 2012), affecting crop productivity. Biotic factors that constrain maize productivity are mostly the result of a negative interaction between plant and another organism. So, the occurrence of diseases and pests can significantly affect the potential productivity of maize (Sangoi et al. 2000; Napoleão et al. 2013). On their turn, abiotic factors, such as drought, temperature and solar radiation, can be even more striking (Heinemann et al. 2008). Besides, in most agricultural areas, productivity is limited by the availability of nutrients in soil. In order to obtain high yields in most crops, as is particularly true for maize, it is necessary to supply mineral fertilizers to the soil. Nitrogen rates recommended for this crop in Brazil may reach values up to 200 kg ha⁻¹, depending on the amount of organic matter present in the soil (Amado et al. 2002; Sangoi et al. 2007). The input of fertilizers, especially nitrogen in non-leguminous crops, is one of the most expensive practices in agriculture, besides represent an environmental hazard. The environmental damage caused by the excessive use of fertilizers involves soil erosion, increased concentrations of nitrate in surface freshwater and groundwater, loss of nitrate by leaching, ammonia volatilization and emissions of nitrous oxide via denitrification (Bijay-Singh et al. 1995; Kennedy et al. 2004). Therefore, although fertilization is necessary, it can represent economic and environmental risks.

As a consequence, researches looking for alternatives that can reduce the use of fertilizers and other chemical inputs in agriculture, without compromising crop productivity, have been developed over the years. One of the most promising technologies is the exploration of soil beneficial microorganisms, since they can play an important role in nature, due to the participation in many biochemical processes. Furthermore, microorganisms play key roles in promoting plant growth by means of different mechanisms, ameliorating both biotic and abiotic stresses (Kadioglu et al. 2012; Maheshwari 2013). In this sense, rhizosphere-associated bacteria stand out as the microorganisms of greatest potential for biotechnological exploration and application.

Although this great importance of microorganisms, the number of microbial groups known and described represents only a small fraction of the microbial diversity found in nature. Soil microbial community has high genetic and functional diversity, and this is as wide as unknown. Knowledge about soil microorganisms is limited in part by the inability to study soil microorganisms by traditional cultivation techniques. It is estimated that one gram of soil may contain between 2000 and 8.3 million microorganisms, representing thousands of species (Gans et al. 2005; Schloss and Handelsman 2006). But approximately only 1% of the soil bacterial population can be cultured by standard laboratory practices (Kirk et al. 2004). In order to assess the bacterial diversity of maize in soils on South Brazil, Roesch et al. (2007) estimated that 1 g of soil contains about 52,000 species. The density of different groups of microorganisms varied depending on soil characteristics and climatic conditions of each environment.

Molecular fingerprinting techniques based on DNA analysis extracted directly from environmental samples, without prior multiplication of bacterial cells have opened a new dimension of studies in microbial ecology. Methods that use molecular manipulation of nucleic acids (DNA or RNA) extracted directly from environmental samples, including DGGE (*Denaturing Gradient Gel Electrophoresis*) or TGGE (*Temperature Gradient Gel Electrophoresis*) (Cahyani et al. 2004; Ning et al. 2009), T-RFLP (*Terminal Restriction Fragment Length Polymorphism*) (Wolsing and Priemé 2004; Stralis-Pavese et al. 2006), ARDRA (*Amplified Ribosomal DNA Restriction Analysis*) (Mittal and Johri 2008), ARISA (*Automated Ribosomal Intergenic Spacer Analysis*) (Cherif et al. 2008; Zancarini et al. 2013), SSCP (*Single-Strand Conformation Polymorphism*) (Bahrathkumar et al. 2008; Castellanos et al. 2009), Microarrays (Ning et al. 2009; Val-Moraes et al. 2011) and DNA cloning and sequencing (Garbeva et al. 2008; Campos et al. 2013) are some of techniques that are used in the cultivation-independent study of microbial community.

With the development and application of molecular genomic tools, the study of non-culturable microbial communities (metagenomics) became possible and has contributed significantly to a better understanding of microbial ecology in various ecosystems. Usually these techniques DNA fingerprints that allow multiple samples analysis to study spatial and temporal variation in the composition of rhizosphere and soil bacterial communities (Muyzer and Smalla 1998). However, all of these approaches have advantages and disadvantages that need to be considered when selecting the method to be used.

7.2 Plant Growth Promoting Rhizobacteria (PGPR) and Maize

Rhizobacteria are free-living bacteria that live close to plant roots. Diverse communities of bacteria inhabit rhizosphere and plant tissues and those bacteria play different roles, influencing plant health and growth. Generally, interactions between plants and microorganisms can be classified as neutral, deleterious and beneficial (Dobbelaere et al. 2003; Doornbos et al. 2012). Beneficial interactions generally involve a group of soil and rhizosphere free-living bacteria colonizing roots in a competitive environment and exerting a beneficial effect on plant growth (Glick 1995; Kirk et al. 2004; Bakker et al. 2007). These bacteria that may promote a beneficial effect on plant growth are collectively named plant growth promoting rhizobacteria (PGPR) (Kloepper and Schroth 1978).

Recently, Maheshwari (2011, 2012, 2013) has elaborated different aspects of PGPR in a series of publications. PGPR can be divided into three groups (Maksimov et al. 2011). The first group is composed by free-living bacteria that specifically interact with plants under favorable conditions. The second group comprises the rhizospheric species localized in soil zones adjacent to roots or on the surface of plant leaves. The third group belongs to the bacteria able to associate with certain tissues of plants by penetrating them through intercellular space (endophytes).

Representatives of the third PGPR group may have advantage over the free-living rhizobacteria. The location inside the plants provide to the endophytes an environment protected from adverse factors, such as soil pH, oxygen pressure and water potential, conditions which may limit populations found on the surface of roots or on rhizosphere soil (Vessey 2003; Choudhary 2012; Mercado-Blanco and Prieto 2012).

Plants can be simultaneously colonized by a wide variety of bacteria and many microorganisms that are found in large number have been characterized as dominant species. In the last decades, it have been reported that maize can establish rhizospheric or endophytic associations with different genera of PGPR, such as *Arthrobacter*, *Azospirillum*, *Achromobacter*, *Bacillus*, *Burkholderia*, *Gluconacetobacter*, *Herbaspirillum*, *Klebsiella*, *Paenibacillus*, *Pseudomonas* and *Rhizobium*. (von der Weid et al. 2002; Hamdia et al. 2004; Rosenblueth and Martínez-Romero 2004; Alisi et al. 2005; Mehnaz and Lazarovits 2006; Rai et al. 2007; Zemrany et al. 2007; Vyas and Gulati 2009; Canellas et al. 2013).

The analysis of microbial communities by cultivation-based methods provides limited information, because most of the existing microorganisms are not likely to be isolated. As stated earlier, with recent advances in genomics and sequencing technologies, microbial community analyses using culture-independent molecular techniques have initiated a new era in the understanding of microbial ecology. In an investigation of rhizobacteria communities from maize plants, using molecular tools, Roesch et al. (2008) found that the majority of the sequences were classified within the Proteobacteria division, with α -proteobacteria and β -proteobacteria being the most abundant in the rhizosphere soil. Also Chelius and Tripplet (2001), studied bacterial community associated with maize roots, found that the α -proteobacteria was the dominant division. In a culture-based study of bacterial diversity within rhizosphere soils and roots of maize, Arruda et al. (2013) found six bacterial divisions: γ -proteobacteria (66.44 %), β -proteobacteria (20.55 %), α -proteobacteria (2.40 %), Firmicutes (1.37 %), Actinobacteria (2.05 %) and Bacteroids (1.37 %) (Fig. 7.1). Another studies suggested that proteobacteria is the most common of the dominant populations found in the rhizosphere of different plant species (Buckley and Schmidt 2001; Schmalenberger and Tebbe 2003; Sun et al. 2008; Rampelotto et al. 2013). The results indicate that these groups are important members of the rhizosphere communities on soil. Although many microorganisms have been detected in association with maize plants, the number and richness of rhizobacteria still remains unknown.

Regardless the methodological limitations, rhizobacteria populations on maize are still studied via culture-based methods, associated or not with culture-independent studies. Indeed, plant species-specific rhizosphere communities have been reported. In fact, Pereira et al. (2011) analyzed the bacterial diversity associated to maize roots though culture-dependent and culture-independent methods. The culturable methods revealed that the predominant group on the roots was Firmicutes, mainly of *Bacillus* genus. Only two genera within γ -proteobacteria division, *Enterobacter* and *Pseudomonas* were found in the culture collection, while the molecular assay showed that *Enterobacter*, *Erwinia*, *Klebsiella*, *Pseudomonas* and *Stenotrophomonas* have been predominant in this group. Montañez et al. (2012) characterized

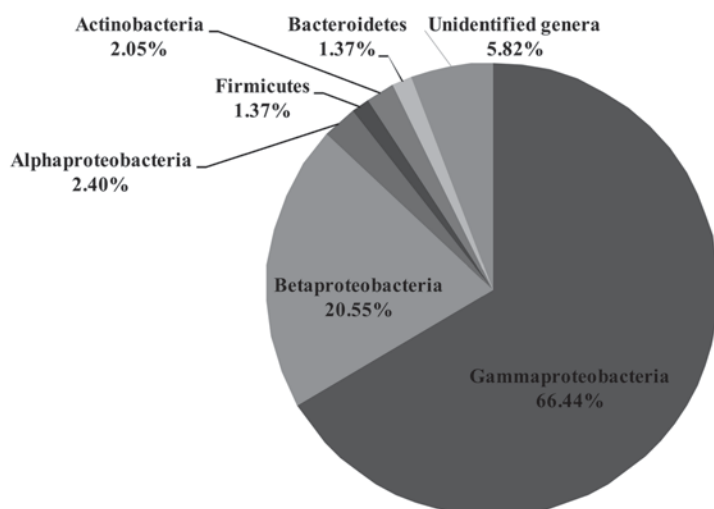


Fig. 7.1 Taxonomic affiliations for roots and rhizosphere soil through analysis of 16S rDNA sequences. (Arruda et al. 2013)

cultivable PGPR and observed a high diversity of bacterial communities, including the genera *Rhanella*, *Pantoea*, *Rhizobium*, *Pseudomonas*, *Herbaspirillum*, *Enterobacter*, *Brevundimonas* and *Burkholderia*. associated with maize cultivars.

Members of the *Burkholderia* genus, belonging to β -proteobacteria division, are considered to be beneficial to the plant by promoting plant growth and biocontrol (Li et al. 2002; Bevivino et al. 2005; Pandey et al. 2005; Vial et al. 2007). Bevivino et al. (1998) observed that strains of *B. cepacia* act against *Fusarium* sp. and, simultaneously, can also stimulate maize growth under iron-limited conditions via siderophore production. According to Di Cello et al. (1997), *Burkholderia* sp. may represent more than 3.5% of the total cultured microbiota from the maize rhizosphere. Besides, Payne et al. (2005) revealed an even higher diversity of uncultured *Burkholderia* species on the maize rhizosphere. Thus, many works have shown that *Burkholderia* sp. is common and abundant in maize plants (Dalmastri et al. 2003; Salles et al. 2006; Suárez-Moreno et al. 2012; Ikeda et al. 2013; Arruda et al. 2013).

Among the pool of microorganisms, bacteria belonging to the genus *Azospirillum* are well-known as PGPR and the beneficial effects to the maize crops have been widely investigated. During the past few decades, *Azospirillum* has been applied to the cultivation of maize and others cereal crops (Pedraza et al. 2009; Hungria et al. 2010). Several modes of action to benefit plants are implicated, especially the synthesis of the phytohormones, such as the indole-3-acetic acid (Spaepen et al. 2007; Masciarelli et al. 2013). Experimental data have shown that inoculations with *Azospirillum* can improve the crop yield. Ferreira et al. (2013) showed that the *Azospirillum brasilense* increased the grain production about 29% compared to nitrogen fertilization alone. This genus colonizes maize irrespective of the soil or geographic location and can be considered as a dominant maize rhizosphere

microorganism (Verma et al. 2011). However, the pattern of root colonization by *Azospirillum* seems to be strongly influenced by host plants. *A. brasilense* and *A. lipoferum* have been found frequently on the rhizosphere of maize (Coninck et al. 1988), while *A. amazonense* has been demonstrated a low occurrence in this culture (Azevedo et al. 2005). According to Baudoin et al. (2009), rhizobacterial community can also be changed, directly or indirectly, by PGPR inoculation. Using the automated ribosomal intergenic spaces analysis (ARISA) to access bacterial diversity, the authors noted that the inoculation of maize seeds with *A. lipoferum* CRT1 caused a shift in the structure of the indigenous rhizobacterial community at 7 and 35 days. It was credited by the inoculation itself, which resulted in the input of a large number of *Azospirillum* cells, directly altering the composition of the rhizobacterial community. At the second sampling, the inoculation has changed root physiology and exudation patterns, which in turn resulted in microbial selection. Now-a-days, in Brazil, *A. brasilense* strains are being used as commercial inoculants in maize, as well as in wheat and rice, resulting in yield increases of up to 25 % (Hungria et al. 2010).

7.3 Factors Influence on Diversity of Rhizobacteria

The rhizosphere is the environment of soil surrounding and influenced by plant roots, and the rhizoplane comprises the plant root surfaces strongly adhering soil particles. In the rhizosphere, very important and intensive interactions are taking place between the plant, soil and microorganisms. It is known that the microbial populations in the rhizosphere are higher in comparison to the bulk soil on different plants (Kowalchuk et al. 2002; Sanguin et al. 2006). Biological interactions and the physio-chemical properties of this region create different growing condition for microorganisms in relation to root free soil (Bais et al. 2006). However, in a study of microbial diversity in the rhizosphere of maize plants, García-Salamanca et al. (2012) reported a decrease in the richness of bacterial communities from bulk soil to the rhizosphere, when culturable bacteria were analyzed. This means that rhizosphere can be highly selective and only a few microbial populations benefit from this environment, becoming dominant.

The bacterial community structure in the rhizosphere is influenced by a variety of biotic and abiotic factors. However, multiplicity of microorganisms cope ecological stress through various modes. Maheshwari et al. (2012) have described amelioration of various abiotic stresses by PGPR. The structural and functional diversity of rhizosphere populations is supposed to be affected by soil type, physico-chemical properties, differences in root exudation and rhizodeposition in different root zones and in relation to plant species, growth stage, cultural practices, such as tillage and crop rotation and other environmental factors (Smalla et al. 2001). Furthermore, all these factors can also directly influence rhizobacteria activity and probably their effect on plant growth and the dynamics of root microbial community. Soil type represents an important factor influencing the structure of microbial

communities. Many researchers have provided evidence that soil type can be an important determinant of the composition of microbial rhizosphere communities (Chiarini et al. 1998; Fang et al. 2005; Canbolat et al. 2006; Wang et al. 2012a; Cotta et al. 2013). Various physical and chemical characteristics of soil such as particle size distribution, pH, organic matter content, salinity, texture, concentration of nutrient elements, seasonal effects, and the management practices like irrigation, tillage and fertilizer application can affect microbial community structure. These characteristics have been reported as the major factors affecting the bacterial composition of the rhizosphere (Garbeva et al. 2004; Zhong et al. 2010; Islam et al. 2011; Shi et al. 2013). Garbeva et al. (2008) observed differences in the grouping of PCR-DGGE fingerprints obtained in response to four plant species and soil with different land use history. These authors suggested that factors, plant type and soil texture, showed clear effects on the structure of total bacteria, *Pseudomonas* and *Bacillus* community.

Other studies have shown that soil type can have influence on natural communities at both inter- and intra-specific levels in the rhizosphere microbiota of maize plants. Dalmastri et al. (1999) demonstrated that the soil type was clearly the dominant factor in affecting the genetic diversity of maize root-associated *B. cepacia* populations, and Bashan et al. (1995) found that populations of *A. brasilense* was largely influenced by soil texture and composition. Moreover, Araújo da Silva et al. (2003) assessed the diversity of the *Paenibacillus* species from the rhizosphere of four different cultivars of maize from two distinct Brazilian soils. DNA sequences closely related to *P. macerans* and *P. azotofixans* were found in all samples. However, other sequences were very scarce and the *Paenibacillus* fingerprints generated via semi-nested PCR followed by DGGE showed a clear distinction between the maize grown in each soil type. This differentiation was not observed among cultivars. Thus, these results suggested that soil type, instead of maize cultivar type, was the overriding determinative factor that influenced the structures of the *Paenibacillus* communities in the rhizospheres. All of these results showed that soil characteristics may benefit the survival of specific microbial populations.

Microbial communities have been associated with characteristics of soils as textures, nutrients content, salinity and soil pH. Among these factors, soil pH have been considered the most influential on the bacterial community in the soil rhizosphere (Fierer and Jackson 2006; Lauber et al. 2008; Wang et al. 2012b). Rousk et al. (2010) found a strong correlation between soil pH and the bacterial diversity. The authors suggested that the reason for this effect is probably due to the narrow pH ranges for optimal growth of bacteria. Soil pH is an important factor affecting the functioning of soil microbial communities in soils. However, according to these authors, one limitation is that it is not known whether the communities are structured directly or indirectly by soil pH. In other words, it is not known whether pH itself is the factor affecting these communities, or whether pH may be indirectly related with other environmental factors complex in nature.

Although soil characteristics provide a great influence on microbial communities, root exudates have been shown to strongly influence the soil microbial community. Microorganisms that inhabit the rhizosphere emit chemical signals that are

recognized by plants and then addressed through the release of chemical compounds in the form of root exudates. The effect of root exudates on the rhizosphere microbial community is likely to be stimulatory or inhibitory. Studies have shown that for maize, the bacterial community structure can differ quantitatively, by changing microbial densities with plant developmental stage and root location (Baudoin et al. 2002). In another work of the same group, Baudoin et al. (2003) evaluated bacterial communities from bulk and rhizosphere soil from maize plants enriched with a mixture of artificial exudates (citric, lactic, succinic and glutamic acid, glucose, fructose, saccharose, alanine and serine). Artificial exudates significantly increased bacterial densities, indicating that structural manipulation of rhizobacteria communities can be mediated by exudates. Recent studies have demonstrated how particular root exudates can attract specific microbes. Chandra et al. (2007) in-vitro study suggested significant increase in bacterial population in rhizosphere due to root exudates. A work by Neal et al. (2012) demonstrated that maize plant roots selectively secreted benzoxazinoids and recruits rhizobacteria *P. putida* to the root. Through the release of a wide variety of compounds, it is suggested that roots regulate the soil microbial community, facilitate beneficial plant-host interactions and change the chemical and physical properties of the soil (Kandeler et al. 2002; Nardi et al. 2002).

The composition of root exudates varies depending on the plant species and the stages of plant development. Cultivation-based and culture-independent methods indicated that plant type is indeed one of the factors influencing the structure of microbial communities. Lawongsa et al. (2008) reported clear differences between total bacterial communities from the rhizospheres of two different plant hosts. This results using ARDRA technique showed that two plant species, rice and maize, selected to two distinct *Pseudomonas* populations. Previously, Brusetti et al. (2004) compared the effect of transgenic Bt 176 maize and non-transgenic plants on the rhizosphere bacterial community and found differences between the two plant types. A significant clustering by principal component analysis suggested that root exudates may be the determining factor in the selection of different bacterial communities. When investigated how maize plants influence the diversity of microbial communities in soil, García-Salamanca et al. (2012) found that plants exerted selective pressure on the bacterial communities, causing enrichment of γ -proteobacteria in the rhizosphere, a group of microbes that are chemotactically attracted by maize roots exudates. This indicates that plants alter the composition more than the abundance of microorganisms, suggesting that differences in composition of root exudates may be responsible for these changes (Picard and Bosco 2008; Aira et al. 2010).

Likewise plant roots results in significant impacts, for example, plant age also influence the composition of the indigenous microbial communities. In a study of *B. cepacia* population naturally occurring in the rhizosphere of maize, Di Cello et al. (1997) observed that microbial genetic variability was higher in the first stages of plant growth and that this rhizosphere bacterial population decreased corresponding to that of time. In young plant roots, however, it is thought that the rhizosphere bacterial populations are dominated by r-strategists, which are species with fast growth rates and capacities to utilize simple substrates (Dey et al. 2012). Further,

young plants provide the highest amount of organic carbon available for microbial growth. It has been hypothesized that the diversity of microorganisms present in different plant rhizospheres, and especially their functional abilities, may be linked to these variations in exudates (Miller et al. 1989). Production of root exudates can be highly variable among individual plants, depending on plant developmental stage and even be able to shape distinct rhizobacteria communities for different genotypes (Micallef et al. 2009). Gomes et al. (2001) also demonstrated that the rhizosphere effect showed a reduced complexity of bacterial diversity for young plants compared to samples taken from mature maize plants, and that this was possible due to root exudates found to be most pronounced for young roots. However, sequencing of clones indicated that the dominant population found at all three plant growth stages can be assigned to *Arthrobacter* populations. Other researchers have reported that the specific structure and diversity of the rhizosphere varies among plant species and over time (von der Weid et al. 2000; Marschner et al. 2004; Herschkovitz et al. 2005).

Soil rhizobacteria communities play important roles in agro-ecosystems, in nutrient cycling and can be affected by agricultural management practices. Numerous studies have investigated the effects of agricultural management practices on soil total microbial diversity and specific groups of microorganisms on different plant-soil systems (Clegg et al. 2003; Spedding et al. 2004; Peixoto et al. 2006; Reeve et al. 2010; Silva et al. 2010; Souza et al. 2013). It is known that the structure of the soil microbiome is influenced by altering crop rotations, tillage and inputs. Ceja-Navarro et al. (2010) analyzed the effect on soil bacterial communities of zero tillage (ZT) under maize monoculture compared to a ZT system under wheat monoculture. Phylogenetic groups found exclusively in wheat were Pseudomonads and Burkholderiae. They found that zero tillage in soil cultivated with maize strongly reduced microbial diversity compared to soil with a monoculture of wheat. The impact of tillage and crop rotations (Wheat/maize) on microbial community structure was assessed by using 454 pyrosequencing of 16S rRNA gene. Recently, Navarro-Noya et al. (2013) observed that zero tillage most affected the bacterial communities and abundance of Actinobacteria, β -proteobacteria and γ -proteobacteria was correlated to the total organic carbon and clay content of soil. Wheat/maize rotation and crop monoculture did not affect richness, diversity and total abundance of bacteria. In a long-term agricultural experiment with two tillage systems and three crop rotations systems studied in the South of Brazil, Quadros et al. (2012) found that the microbial diversity was higher in the zero tillage system in four taxonomic levels and demonstrated that, at all taxonomic levels, the diversity was significantly higher in crop rotation without legumes, suggesting that the crop system based on cereals stimulates microbial diversity on the soil. Soil under zero tillage may result in different conditions such as higher organic matter content and moisture, counterparted by negative effects such as an increase on soil density, which could affect the structure of soil microbial community (Helgason et al. 2009).

The knowledge about the shifts of microbial community structure and diversity following fertilizers applications are still not unanimous. Soil bacteria seem to be more sensitive to fertilizer application and excessive use of fertilizer harms micro-

bial population (Aeron et al. 2011). According to Tambong and Xu (2013) a more diverse community structure of both γ -proteobacteria and *Pseudomonas* sp. was found in nonfertilized than fertilized (additional N application) soils irrespective of plant types under cultivation. Earlier, Ge et al. (2008) observed changes in bacterial community structures in corn fields under long-term fertilization regimes. The organic manure (OM) and phosphorus and potassium (PK) treatments showed a higher richness and diversity when compared to other treatments, such as the ones that received chemical nitrogen fertilizers. In other words, N fertilizer could be considered as an impact factor on soil microbial communities that results in the decrease on its diversity. Lupwayi et al. (2012) investigated the soil microbial response to different rates of nitrogen fertilizer on barley and corn crops, in different tillage, and verified that the functional diversity of soil bacteria was not reduced even at the highest N applications on corn crop. So, understanding the mechanisms that regulate microbial community structure of various management practices may lead to the development of best practices for improving soil fertility and crops productivity.

7.4 Molecular Techniques to Assess Bacterial Diversity

The use of molecular methods for identification and detection of microbes is making it possible to uncover phylogenetic relationships within microbial communities. A set of methods, including culture-based and culture-independent techniques, provide a comprehensive view of the microbial community in different environments (Edenborn and Sexstone 2007; Rani et al. 2008; Jin et al. 2011).

It is estimated that only 1 % of the microorganisms is detected in laboratory culture, depending on the composition of the culture medium and on the growth rates of the microbial community populations (Kirk et al. 2004; Hirsch et al. 2010). In opposition to this inherent limitation of culture-based methods, culture-independent approaches indicate the presence of innumerable uncultivated species. An increasing number of culture-independent methods are being proposed for microbial community analysis based on the extraction, quantification, and identification of molecules from environmental samples that are specific to certain microorganisms or microbial groups. Because of that, it is believed that molecular tools allow to access to the entire microbial community genome and permit quick and simultaneous analysis of multiple samples.

On the other hand, culture-based approaches do not necessarily provide comprehensive information on the composition of microbial communities, but are extremely useful for understanding the physiological potential of isolated organisms (Amann et al. 1995; McCaig et al. 2001). Using culture-based approaches is the only way to isolate and study strains with biotechnological potential usage, for instance. Besides, some studies reveal that, at least in some circumstances, culturable bacteria are the largest, most active and ecologically relevant portion of the soil bacterial community and, because of that, are more sensitive to soil disturbance than the whole community (Ellis et al. 2003). The culture-based approach can also

capture more members of low abundance on soil, the so called rare-biosphere, than culture-independent pyrosequencing (Shade et al. 2012). Thus, both approaches, culture-based and culture-independent, allow the characterization of the microbial diversity at different levels and can be considered complementary.

Most of the molecular techniques for the detection, identification, and classification of bacteria are based on differences in the composition of the ribosomal genes. Analysis using ribosomal genes, especially 16S rRNA were first used by Woese et al. (1990) and showed that this molecule was an excellent molecular marker for phylogenetic studies. There are regions on the 16S rRNA that are highly conserved and others that comprise variable sequences. Comparing the differences in the base sequence of 16S rRNA gene is, therefore, an excellent mean to study evolutionary changes and phylogenetic relatedness of microorganisms (Janssen 2006; Vasileiadis et al. 2012). The advantage of use of 16S rRNA gene is that it can be used to identify isolated bacteria strains as well as to access the diversity of all bacteria communities. Sequencing this gene allows the identification of microorganisms at the genus and/or species level. As an example of its use, Garbeva et al. (2008) sequenced this gene and established phylogenetic relationships among members from microbial communities of maize rhizosphere soil.

PCR fingerprinting is used to distinguish differences in the genetic makeup of microbial populations from different samples and can be accomplished by several different methods (Hirsch and Sigmund 1995; Kozdrój and van Elsas 2000; Okubo and Sugiyama 2009). There are numerous PCR-based techniques that can be applied to the study of biodiversity (Table 7.1). The most common PCR fingerprinting techniques in use for characterizing soil microbial communities are DGGE/TGGE (Garbeva et al. 2003; Zhao et al. 2012), T-RFLP (Sessitsch et al. 2001; Noll et al. 2005; Edel-Hermann et al. 2008) and ARDRA (Mittal and Johri 2008; Estrada-de los Santos et al. 2011). These fingerprinting approaches have been considerably effective on detecting differences among microbial communities and can be used as additional methods to separate PCR products that are initially of the same length, into a greater number of bands or operational taxonomic units (OTUs) that are then used for community comparisons. Most commonly, primers that target regions in the 16S rRNA, which is conserved for all eubacterial species, are used. However, other functional genes, such as *nifH* (Soares et al. 2006), can be used, depending on the objective of the research.

DGGE or TGGE are two similar methods for studying microbial diversity. Both techniques prescribe a parallel gradient of denaturing conditions along a polyacrylamide gel (Muyzer and Smalla 1998). The difference is that in DGGE the denaturant agents are typically urea and formamide, while in TGGE it is temperature. However, the fingerprints obtained through those molecular tools do not give direct taxonomic information, for which later analysis has to be done to assigned the population fingerprints, and this is their main limitation.

T-RFLP is a culture-independent technique for assessing microbial community diversity using specific restriction enzymes. One or both primers can be labeled with fluorochrome and the PCR products are digested with restriction enzymes to reveal terminal restriction fragment polymorphisms which are then separated

Table 7.1 PCR-based methods used to study soil microbial diversity

Method	Principles	Advantages	Disadvantages	Reference
<i>DGGE/TGGE</i>	Separation of DNA fragments of the same length but with different base pair sequences. Based on the decreased electrophoretic mobility of a partially melted DNA molecule	Bands can be excised, cloned and sequenced for identification	Can be used for only short fragments Complex communities may appear smeared due to a large number of bands	Muyzer et al. (1993)
<i>T-RFLP</i>	Based on the restriction endonuclease digestion of fluorescently end-labeled PCR products and the fragments are then separated by gel electrophoresis	Sensitive to variation in DNA sequence Enable analysis of a wide array of microbial community	Distinct sequences that sharing a restriction site will result in one band	Kitts (2001)
<i>ARDRA</i>	Method based on restriction endonuclease digestion of the amplified of ribosomal gene	Highly useful for detection of structural changes in simple microbial communities	More applicable to environments with low complexity Different bands can belong to the same group	Wang et al. (2011)
<i>ARISA</i>	The method involves PCR amplification from total bacterial community DNA of the intergenic region between the small (16S) and large (23S) subunit rRNA	Distinguish bacterial strains and closely related species Rapid and sensitive	Similar intergenic space length may lead to underestimations of community diversity	Zancarini et al. (2013)
<i>SSCP</i>	The technique is capable of identifying most of the sequence variations in a single strand of DNA, typically between 150 and 250 nucleotides length	Community members can be identified Helps to identify new mutations	Factors such as mutation and length of fragments can affect the reproducibility of the method	Bahrathkumar et al. (2008)

by gel electrophoresis (Pandey et al. 2007; Schütte et al. 2008). T-RFLP has been widely used for the analysis of bacterial communities in different conditions (Wu et al. 2009; Kim et al. 2010; Fortuna et al. 2011; Sheng et al. 2012) and to assess spatial and temporal heterogeneity and dynamics of bacterial communities in soil (Lucklow et al. 2000; Kuske et al. 2002). T-RFLP has appeared to be more sensitive in resolving bands than DGGE fingerprinting (Sessitsch et al. 2002). On the other hand, DGGE analysis has an important advantage over T-RFLP: amplicons of interest can be excised from the DGGE gel, re-amplified, cloned and sequenced, thereby obtaining taxonomic and/or phylogenetic information about members of the soil community.

The ARDRA technique has been successfully applied to ecological population studies, and has the advantage of provide taxonomic resolution at species level (Heyndrickx et al. 1996). This technique is based on the degree of conservation of restriction sites, which reflects rRNA phylogenetic patterns. ARDRA allows a rapid assessment to the diversity of an environment sample.

The general principle of most molecular fingerprinting techniques is based on the electrophoretic separation of marker gene PCR-amplified fragments from nucleic acids directly extracted from soil samples, due to differences in their nucleotide sequence. In these methods, the microorganisms can be characterized by cloning and sequencing of differentiating bands or by probing. Generally, the choice to select better method will often be influenced by the expertise and the availability of equipment in the laboratory.

Sequencing techniques are widely applied to the study and understanding of microbial diversity. The construction of libraries and sequencing of clones from ribosomal genes is a highly sensitive molecular tool, which allows the detection of uncultured microorganisms, which represents 99% of the total species existent in environmental samples (Leigh et al. 2010). However, cloning and conventional sequencing methods are time consuming and limit the number of samples that can be processed. More recently, the development of microarrays and next-generation sequencing made possible the massive parallel sequencing of environmental samples.

As previously discussed, the study of the microbial diversity has undergone a revolution with the establishment of metagenomics, defined as the investigation of collective microbial genomes retrieved directly from environmental samples, without relying on cultivation or prior knowledge of the microbial communities (Leveau 2007; Schloss and Handelsman 2008; Sharma et al. 2008). Thus, metagenomics approach can circumvent the limitations of culture-based methods and, especially when associated to most modern methods, such as high-throughput sequencing approaches and microarray techniques, provides a wider access to microbial communities. An example of these new tools is the 454 sequencing technology, a pyrosequencing platform which allows massive parallel sequencing of hyper variable regions of 16S rRNA genes and offers coverage of microbial diversity two to three orders of magnitude higher than typical Sanger sequencing method (Fakruddin et al. 2013). In farming systems, pyrosequencing has been used to evaluate the effect of conventional and organic systems on soil rhizobacterial communities (Liu et al. 2012; Yang et al. 2013), for instance.

Microarray application for soil microbial ecology has been studied recently (Sanguin et al. 2008; Val-Moraes et al. 2011; Chapman et al. 2012; Xiong et al. 2013). Likewise pyrosequencing, this method is reported to reveal higher soil bacterial phylogenetic diversity compared to more conventional cloning or sequencing methods (DeSantis et al. 2007). The application of microarrays has proved to be a useful tool in the advancement of microorganism characterization in natural habitats. Val et al. (2009) used commercially available DNA microarrays containing genome-wide spotted oligonucleotides encompassing *Bacillus subtilis* or *Streptomyces coelicolor* genomes to monitor rhizobacterial communities of conventional and transgenic maize in three different agricultural soils. The authors found no significant differences when transgenic and non-transgenic rhizosphere DNAs were hybridized to either type of microarray, suggesting that the Cry endotoxin released from the transgenic Bt maize does not affect the bacteria present in those rhizospheres. In spite of the absence of significant differences among the treatments, those microarrays proved to be reliable and sensitive, detecting as little as five copies of a particular gene in the sample. In addition to identifying the diversity of communities, such arrays can give some indication about the relative abundance of different taxa in different treatments or environments (Sanguin et al. 2006; DeAngelis et al. 2009).

7.5 Conclusion and Future Perspectives

Rhizosphere is a special environment within soil, which has a rich pool of bacteria. Bacteria are the most abundant microorganisms that reside in rhizosphere and a special class of bacteria, denominated as PGPR, influences the plant growth by a variety of direct and indirect mechanisms, in a wide range of economically important crops, including maize. Considering the economic and nutritional importance of maize, the increasing demand for grains and the consequent increasing demand for fertilizers, the use PGPR stands out as a promising, inexpensive and environmentally friend technology. The search for effective PGPR strains and the investigation of their modes of action are increasing at a rapid pace as efforts are made to exploit them commercially as biofertilizers. Meanwhile, advances on molecular techniques are providing the characterization of uncultured microorganisms that can also be important members of microbial communities. The understanding of how biotic and abiotic factors can influence rhizobacterial diversity is of fundamental importance to select agricultural practices that modify such factors in order to favor beneficial microorganisms and increase crop productivity, as a consequence. Selecting potentially effective PGPR, understanding the diversity bacteria in the rhizosphere and identifying the factors that have impact on microbial community structure, will facilitate the exploration of PGPR as a reliable component in the management of sustainable agricultural system.

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Chapter 8

Transgenic Cotton and Its Impact on Microbial Diversity

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Abstract Introduction of GM crops into agricultural production systems increased public concern and renewed interest in research on the possible environmental consequences of growing GM crops including human health and ecosystem functioning. Globally, *Bacillus thuringiensis* (*Bt*) cotton occupies 15 million ha which comprised 43 % of the total cotton area of 35 million ha. *Bt* cotton was developed by incorporating the *cry* gene of the soil bacterium *Bacillus thuringiensis*. This gene expresses the protein endotoxin (*Cry*) that has insecticidal activity against the common cotton lepidopteran insect pests. While the benefits of *Bt* cotton are well known, there is a wide spread concern about growing transgenic cotton. This stems from the fact that the *Bt* toxin produced in leaves, stems and roots of *Bt* cotton is introduced in soil which might affect general soil health. Several workers have studied the effects of transgene products and transgenic cotton on the soil biological properties. Quite a few studies assessed the risk of growing *Bt* cotton on flora and fauna in diverse agro-ecosystems. This chapter attempts to review the work done so far related to growing transgenic *Bt* cotton on the soil microbial diversity and other related soil functions.

8.1 Introduction

Genetically modified (GM), genetically engineered, or transgenic crops refer to plants produced by the insertion of specific pieces of nucleic acids into the plant's DNA using recombinant DNA technology (i.e., *Agrobacterium*-mediated transformation or direct gene transfer methods) (Griffiths et al. 2005). This biotechnological approach allows genes to be introduced into a plant genome from any source (i.e., plant, animal, bacterial and fungal) resulting in potential transfer of a wide range of genetic resources between unrelated species, a major difference compared to traditional plant breeding that is limited to exchange of genetic material only between sexually compatible close relatives of a given plant (Mirkov 2003). Transgenic plants that show herbicide tolerance, resistance to viral, bacterial and fungal

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Table 8.1 Commercial *Bt* crops and genes expressed by them

Crops	Gene	Target pest
Cotton	<i>cry1Ac</i>	Bollworm
Cotton	<i>cry2Ab</i>	Bollworm
Corn	<i>cry1Ab</i>	European corn borer
Corn	<i>cry9C</i> (discontinued)	European corn borer
Corn	<i>cry1Ac</i>	European corn borer
Corn	<i>cry3Bb</i>	Corn rootworm
Corn	<i>cry1F</i>	European corn borer, southwestern corn borer, fall armyworm and black cutworm
Potato	<i>cry3Aa</i> (discontinued)	Colorado potato beetle
<i>Crops under development</i>		
Cotton	<i>cry1Ac</i> + <i>cry2Ab</i>	Bollworm
Cotton	<i>cry1Ac</i> + <i>cry1F</i>	Bollworm and fall armyworm
Cotton	<i>Vip3A</i>	Bollworm and fall armyworm
Corn	<i>cry34Ab/35Ab</i>	Corn rootworm

diseases, insect resistance, improved product quality and superior agronomic properties were introduced in the mid-1990s. Plant species that have been genetically engineered include: maize, tomato, cotton, soybean, oilseed rape and to a lesser extent potato, squash, beet, rice, flax, papaya and cichorium.

Worldwide 12 major crops such as soybean, maize, cotton, canola, potato, sugar beet, alfalfa, papaya, squash, tomato, poplar and sweet pepper have been genetically modified, commercially cultivated (James 2010). Soybean is the leading GM crop occupying 75.4 m ha, followed by maize (51.00 m ha), cotton (24.7 m ha) and canola (8.2 m ha). Initially, six countries namely USA, China, Canada, Mexico, Australia and Argentina took up cultivation of GM crops, this number increased to 29 in 2011. Growing awareness of GM crops and acceptance by farmers resulted in an increase in global area under GM crops from 1.7 m ha in 1996 to 160 m ha in 2011 (James 2010). Presently, two principal transgenic technologies dominate the market: (i) herbicide-tolerant (HT) crops and (ii) insect resistant crops (*Bt* crops). *Bt* crops increased productivity and reduced insecticide usage, providing additional benefits to human health and the environment (Table 8.1).

There has been a strong debate on the safety of genetically modified plants ever since the introduction of transgenic plant products into the market. This debate is still very much alive and several issues were raised, including the safety of transgenic food and the environmental impact of transgenic plants (Schubert 2002; Dale et al. 2002; Liu et al. 2005). However, the potential development of resistance to the *Bt* toxin by insect pests and the indirect damage of *Bt* toxins to non-target species are major concerns related to their use. Introduction of GM crops took place in 1996, when biotechnology-derived herbicide tolerant (HT) and insect-resistant traits were launched into the market in soybean, cotton, corn, and canola. These 'input traits' were designed to benefit the farmer directly and aimed to increase productivity per hectare, reduce agrochemical use, decrease production costs, have greater flexibility and efficiencies in production regimes, and improve grower health (Hossain et al. 2004; Huang et al. 2005).

8.2 Mechanisms of Transgenic Plants Affect Soil Microorganisms and Functions

With the introduction of GM crops into agricultural production systems public concern increased. This also renewed interest in research on possible environmental consequences of growing GM crops including human health and ecosystem functioning (Sessitsch et al. 2004; Brookes and Barfoot 2005; Marvier et al. 2007). Soil microorganisms are responsible for different key functions in ecosystems as they are involved in many decomposing processes as well as in all major biogeochemical cycles, in the recycling of essential elements. Studies of the impact of genetically modified organisms should therefore, also focus on microbial community functions as they are key elements in a healthy ecosystem (Lamarche and Hamelin 2007). Cultivation of transgenic crops are reported to affect soil functions by direct (transgene proteins) and indirect effects (changes in plant protein, root exudates composition, modification in metabolic pathways). GM crops have the potential to influence soil microbiology through (i) the exudation of transgenic proteins from the root system, (ii) the release of transgenic proteins from broken and dying roots, (iii) the incorporation of above ground plant material into the soil, and (iv) differences in exudation chemistry (Gupta and Watson 2004; Knox et al. 2006; Saxena and Stotzky 2001).

8.3 Indicators of Soil Biological Quality: Why to Measure?

Soil quality is defined as, “the capacity of a soil to function within its ecosystem boundaries to sustain biological productivity and diversity, maintain environmental quality, and promote plant and animal health” (Brady and Weil 1999). Soil quality cannot be measured directly, so we evaluate through indicators. Indicators are measurable properties of soil or plants that provide clues about how well the soil can function. Indicators can be physical, chemical, and biological properties, processes, or characteristics of soils. Soil quality indicators are useful to policy makers to monitor the long-term effects of farm management practices on soil quality; assess the economic impact of alternative management practices designed to improve soil quality by including not only environmental values but also taking into account economic and social factors. Some of the key indicators of soil biological quality are presented in Table 8.2.

8.4 What is *Bt*?

“*Bt*” is short for *Bacillus thuringiensis* it is a soil bacterium occurring naturally. *Bt* was first discovered in 1901 by Shigetane Ishiwatari. In 1911, *B. thuringiensis* was re-discovered in Germany by Ernst Berliner. He isolated the cause of a disease

Table 8.2 Key indicators of soil biological quality

Indicators	Functions
Soil respiration	Soil respiration is a useful index of the overall biological activity in soil and is a critical determinant of ecosystem carbon storage. It reflects the intensity of the soil organic matter decomposition and mineralization and the incidence of the microorganisms in soil, and it is often used for the biomass determination
Fluorescein diacetate hydrolysis (FDA)	FDA hydrolysis assay measures the enzyme activity of microbial populations and can provide an estimate of overall microbial activity in an environmental sample. The assay is considered non-specific because it is sensitive to the activity of several enzyme classes including lipases, esterases, and proteases
Soil microbial biomass (SMB)	SMB is a living pool containing 1–5% of the soil organic matter. Microbial biomass determinations indicate changes in the soil organic matter before they can be detected by measuring total soil carbon making possible its use as an indicator of early changes in soil organic matter content. Microbial biomass consists of both dormant and metabolically active organisms and has been considered as an integrative indicator of microbial significance of soils
Soil urease	Urease plays an important role in the efficient use of urea fertilizer in soils and the changes in urease activity can be used as an indirect indicator of the variation in the pool of potentially available N in a soil
Soil dehydrogenase	The dehydrogenase enzyme activity is commonly used as an indicator of biological activity in soils. This enzyme is considered to exist as an integral part of intact cells but does not accumulate extracellularly in the soil. Dehydrogenase enzyme is often used as a measure of any disruption caused by pesticides, trace elements or management practices to the soil
Phosphatases	Phosphatases are a broad group of enzymes that are capable of catalysing hydrolysis of esters and anhydrides of phosphoric acid. In soil ecosystems, these enzymes are believed to play critical roles in P cycles
β glucosidase	This enzyme plays an important role in soils because it is involved in catalysing the hydrolysis and biodegradation of various β -glucosides present in plant debris decomposing in the ecosystem. Its final product is glucose, an important C energy source of life to microbes in the soil
Arylsuphatases	They are responsible for the hydrolysis of sulphate esters in the soil and are secreted by bacteria into the external environment as a response to sulphur limitation
Soil microorganisms	Microorganisms have double role in relation to soil fertility. On one hand, the microbes are the agents that mineralise and liberate plant nutrients from the organic material. On the other hand, the microorganisms can also be viewed as a collective observer of the soil environment. Since the microbes are in close contact with all three soil phases (Solid, water and air), they can sensitively and rapidly probe responses to soil perturbations
Glomalin content	Indicator of mycorrhizal activity in soil. Reported to involved in soil aggregation

called *Schlaffsucht* in flour moth caterpillars. A unique feature is its produce *crystal* proteins called as “*CRY* proteins” or “Insecticidal *Crystal Protein*” (ICP) that selectively kills specific groups of insects for example Lepidopteran caterpillars (moth and butterflies), Diptera (mosquitoes and black flies), Coleoptera (beetles), and nematodes.

8.5 Mode of Action by ICP

The target organ for *Bt* toxins is the insect larvae’s mid-gut. The mid-gut of the larvae is a simple, tubular epithelium that dominates the internal architecture of the insects. After ingestion by insect’s larvae, the *Bt* δ -endotoxin disrupt of the epithelium in the insect mid-gut. The alkalinity of insect mid-gut (pH 12) dissolves the *Crystals*, releasing the *Cry* pro-toxin where it is cleaved by insect proteases to generate the trypsin resistant core of the active δ -endotoxin. The active toxin traverses the peritrophic membrane to bind receptor of brush border cells of the insect mid-gut. Integration of the toxin into the epithelial membrane, resulted in osmolysis of the cells, and paralysis occurred and dies within 2 days. Different *Bt* strains produce different *CRY* proteins, and there are hundreds of known strains which have identified more than 60 types of *Cry*-proteins that affect a wide variety of insects.

8.6 Persistence of *cry1* Toxins from *Bt* Cotton

During plant litter decomposition, most transgene protein(s) appear to be rapidly degraded. However, some proteins can bind to surface-active particles and reduce their availability to microbes. Sims and Ream (1997) estimated that a potential maximum of 1.6 mg *Cry2A* protein kg⁻¹ soil would result from the incorporation of *Bt* cotton residues into the top soil (Table 8.3). Recently, Sun et al. (2007) reported that the *CryIAb* protein persisted in soil for at least 56 days after incubating a slit loam soil with *Bt* cotton tissues.

8.7 Impact of Transgenic *Bt* Cotton Cultivation

Cotton (*Gossypium* spp.) belonging to the genus *Gossypium* in the family Malvaceae is an important fiber crop of global importance. Cotton is grown in tropical and subtropical regions of more than 80 countries. It is an important source of oil and high quality protein and plays a significant role in the national economy. Besides being the backbone of the textile industry, cotton and its byproducts are also part of the livestock feed, seed-oil, fertilizers, papers and other consumer products. Handling, processing and production of various consumer based products of cotton

Table 8.3 Studies on persistence of Cry proteins in soil

Protein	Experiment	Findings	References
Cry1Ab Cry1Ac Cry3Aa	Soil amended with biomass of <i>Bt</i> cotton	No persistence of proteins in soil; proteins degraded in soil with a half-life of 20 days	Ream et al. (1994)
Cry1Ab Cry1Ac	Soil amended with purified protein or biomass of <i>Bt</i> cotton	Purified protein was detected up to 28 days and the protein from <i>Bt</i> cotton was detected up to 56 days	Donegan et al. (1995)
Cry1Ab Cry1Ac	Soil amended with purified protein or biomass of <i>Bt</i> cotton	Purified proteins and Cry proteins from cotton tissue decreased rapidly, with a half-life of approximately 4 and 7 days, respectively, by ELISA	Palm et al. (1996)
Cry2A	Soil amended with biomass of <i>Bt</i> cotton <i>Bt</i> cotton cultivation	Half-life of bioactivity was estimated at 15.5 days by insect assay Half-life of bioactivity was estimated at 31.7 days by insect assay	Sims and Ream (1997)
Cry1Ac	<i>Bt</i> cotton cultivation	No detectable level of protein in soil for 3–6 consecutive years	Head et al. (2002)

also play an important role in the social and industrial structure. Cotton is long duration crop and it is reported to be attacked by more than 162 species of insect pests including sucking pests, tissue borers and defoliators at various stages of growth, causing losses up to 60%. Cotton is vulnerable to a number of insect species, especially to the larvae of lepidopteran pests. The cotton bollworm complex is a major and serious threat to the cotton, causing potential yield losses across the world and reported that the annual loss of at least US \$ 300 million. High level of insecticide resistance in bollworms necessitates repeated application of insecticides, thereby aggravating the problem of resistance and also leading to heavy expenditure on cultivation and crop failures. Therefore it was important to initiate the development of alternative technologies such as genetic modification to enable plants to resist against the insect attack.

8.8 Transgenic *Bt* Cotton on Pest Control

The era of transgenic cotton began when Perlak et al. (1990) introduced *cry* 1A(b) and *cry* 1A(c) genes into cotton (*G. hirsutum*) plants and transformed plants showed a high level of resistance to *Helicoverpa*. During the field and laboratory tests, it was demonstrated that transgenic cotton is highly effective against neonate larvae of *H. armigera* (cotton bollworm), *H. virescens* (Tobacco budworm), and *Pectinophora*

Table 8.4 Event released for commercial cultivation of *Bt* cotton

Gene	Events	Company/institute
<i>cry</i> I Ac	MON 531	Monsanto company
<i>cry</i> I Ac and <i>cry</i> II Ab	MON 15985	Monsanto company
<i>cry</i> I F	2581-24-236	Dow Agrosiences
<i>cry</i> I Ac	3006-210-23	Dow Agrosiences
<i>cry</i> I Ac	31807/31808	Calgene
Vip 3A(a)	COT 102	Sangenta Seeds
<i>cry</i> I Ac and <i>cry</i> I F	Das-21023-5 * Das 24236-5	Dow Agrosiences
<i>cry</i> I Ab/ <i>cry</i> I Ac		CAAS/Nath Seeds
<i>cry</i> I Ac		NRCPB/UAS Dharwad
<i>cry</i> I E/C, <i>cry</i> I Ac		NBRI/Swarna Bharat Biotechnics Pvt. Ltd
<i>cry</i> I Ac	Event I	IIT Kharagpur/JK Agri-genetics
<i>cry</i> I Ac, CPTi		Mahyco
<i>cry</i> I Ac, CPTi		Nath Seeds, CICR

gossypiella (pink bollworm). The *Bt* gene from the original genetically engineered mother plant was Coker-312. Transferred to advanced cotton cultivars through backcrossing. Later Gene stacking, involving the introduction of more than one gene of similar effects is becoming an attractive alternative for developing durable resistance and for simultaneous and effective control of more than one insect together. For instance, in cotton, Monsanto transgenic event ‘Bollgard-II’ carries two genes viz, *cry* 1Ac (against American Bollworm and *cry* 2Ab (Against tobacco bud worm) (Table 8.4).

Bt cotton occupies globally 15 million ha which comprised 43% of the total cotton area of 35 million ha in nine countries namely USA, Mexico, China, Argentina, South Africa, Colombia, India, and Brazil (ISAAA 2006). GM cotton were developed by incorporating and expressing crystal protein endotoxin (*Cry*) encoded by the *cry* gene of the soil bacterium *B. thuringiensis* having insecticidal activity against the common cotton infecting insects belonging to orders Lepidoptera, Diptera, and Coleoptera (Wallimann 2000). Although there is large-scale adoption of *Bt* cotton by the farmers because of immediate financial gain, there is concern that transgenic *Bt* crops release *Bt* toxins into the environment which affect associated and succeeding crops due to a reduction in soil chemical and biological activities (O’Callaghan et al. 2005; Sarkar et al. 2008).

8.9 Transgenic *Bt* Cotton on Microbial Diversity and Soil Functions

In experiments to evaluate the persistence of Cry1 toxins from *Bt* cotton leaves incorporated into soil microcosms, Palm et al. (1996) found that degradation of the toxin was microbially mediated as suggested by various reports from the Stotzky group (Koskella and Stotzky 1997; Crecchio and Stotzky 1998). Bacterial

Table 8.5 Cry proteins from *Bacillus thuringiensis* on the microbial population and diversity

Protein	Microorganisms	Experiment	Findings	References
Cry1Ac	Culturable bacteria and fungi	Soil with <i>Bt</i> and non- <i>Bt</i> cotton	A significant, increase in numbers in soil with <i>Bt</i> cotton	Donegan et al. (1995)
Cry1Ac	Microbial population	<i>Bt</i> and non- <i>Bt</i> cotton	No adverse effect	Valasubramanian (2001)
Cry1Ac	Composition of soil microbiota	Rhizosphere soils of <i>Bt</i> and non- <i>Bt</i> cotton	More extensive fungal colonization, higher ratios of fungi to bacteria, and different types of fungal spores in soil with <i>Bt</i> cotton	Gupta and Watson (2004) Gupta et al. (2002)
Cry1Ac	Culturable functional bacteria	Rhizosphere soils of <i>Bt</i> and non- <i>Bt</i> cotton	No significant differences in numbers after the growing season	Rui et al. (2005)
Cry1Ac	Functional diversity of microbial communities	Soil with <i>Bt</i> and non- <i>Bt</i> cotton	No adverse effects	Shen et al. (2006)
Cry1Ac	Methylobacteria	<i>Bt</i> and non- <i>Bt</i> cotton	No adverse effects	Balachandrar et al. (2008)
Cry1A	Culturable functional bacteria	Multiple-year (0–5 years) cultivation of <i>Bt</i> cotton	No adverse effects	Hu et al. (2009)
Cry1Ac	Microbial diversities	Soil with <i>Bt</i> and non- <i>Bt</i> cotton	No adverse effects	Kapur et al. (2010)
Cry1Ac	Microbial population and diversity	Soil with <i>Bt</i> and non- <i>Bt</i> cotton	Higher microbial population and diversity in <i>Bt</i> cotton	Velmourougane and Sahu (2013)

community structure was less affected by the *cry1Ab* protein than by other environmental factors, such as plant age or field heterogeneity (Baumgarte and Tebbe 2005). However, undue decrease in microbial community richness with the use of genetically modified cotton is also reported (Dunfield and Germida 2004). Previous studies have shown that the qualitative and quantitative differences in root exudation of *Bt* cotton could strongly influence the structure of microbial communities in the rhizosphere (Oger et al. 2000; Yan et al. 2007). A significant but transient increase in the populations of culturable bacteria and fungi was observed in soil amended with leaves of *Bt* cotton (*Gossypium hirsutum* L.) expressing the *Cry1Ac* protein in comparison to the wild type plant measured by BIOLOG analysis and DNA fingerprinting (Donegan et al. 1995). Higher microbial counts in transgenic cotton grown soil have also been reported by several workers (Shen et al. 2006) (Table 8.5). Head et al. (2002) demonstrated that the amount of Cry1Ac protein accumulated as a result of continuous use of transgenic *Bt* cotton, and subsequent

incorporation of plant residues into the soil by postharvest tillage for 3 to 6 consecutive years is extremely low and does not result in detectable biological activity. Rui et al. (2005) reported that the fortification of pure *Bt* toxin into rhizospheric soil did not result in significant changes in the numbers of culturable functional bacteria, except the nitrogen-fixing bacteria when the concentration of *Bt* toxin was higher than 500 ng/g. The results indicated that *Bt* toxin was not the direct factor causing decrease of the numbers of bacteria in the rhizosphere, and other factors may be involved. Balachandar et al. (2008) studied the diversity richness of Pink-pigmented facultative methylotrophs (PPFMs) present in the phyllosphere, rhizoplane and internal tissues did not differ between *Bt* and non-*Bt*-cotton and reported that there was no evidence to indicate any adverse effects of *Bt* cotton on the diversity of plant-associated methylobacteria.

Hu et al. (2009) reported that there were no consistent differences in the numbers of different groups of functional bacteria between rhizosphere soil of *Bt* and non-*Bt* cotton in the same field. Further, no obvious trends were observed with regard to the numbers of the various groups of functional bacteria with an increasing duration of *Bt* cotton cultivation. Sarkar et al. (2009) concluded from their study that there were some positive or no negative effects of *Bt*-cotton on the soil quality indicators (microbial biomass carbon, microbial biomass nitrogen, microbial biomass phosphorus, total organic carbon, microbial quotient, potential nitrogen mineralization, nitrification, nitrate reductase, acid and alkaline phosphatase activities, Root dry weights, and root volume). Therefore cultivation of *Bt* cotton appears to pose no risk to soil ecosystem functions (Table 8.6). The microbial community structure in soil was not affected by the cropping of *Bt* cotton and the total microbial population and diversity of experimental fields remain quite similar during the cropping of both *Bt* cotton and non-*Bt* cotton (Kapur et al. 2010). Based on the studies conducted at Central Institute for Cotton Research, Nagpur, it was found that growing *Bt* cotton does not affect the soil biological properties (soil respiration, urease activity, dehydrogenase activity, and microbial biomass carbon). The results obtained with culturable microbial population and microbial diversity index analysis further proved that the microbial activity in soil was not affected by the cropping of *Bt* cotton (Velmourougane and Sahu 2013). Cry proteins from *B.thuringiensis* was also not reported to affect the soil invertebrates (Table 8.7). These results suggest that cultivation of *Bt* cotton expressing *cryIAC* gene may not poses ecological or environmental risk.

8.10 Conclusion

A major problem in assessing the impacts of transgenic crops on soil microbial attributes is the lack of baseline information on diverse agro-ecosystems to compare with ecosystems in which transgenic crops were introduced and lack of universally approved approach for carrying out impact assessment of the transgenic plants on soil ecosystem. Genetic modifications have been performed with several

Table 8.6 Effects of *Bt* cotton on microbe-mediated process and functions in soil

Protein	Process/function	Experiment	Findings	References
Cry1Ac	Soil enzymes (Urease, Alkaline Phosphatases, dehydrogenase, phenol oxidase, proteases)	Soil with <i>Bt</i> and non- <i>Bt</i> cotton	No differences in the activities of enzymes	Shen et al. (2006)
Cry1Ac	Selected enzymes	Soil amended with <i>Bt</i> and non- <i>Bt</i> cotton biomass	Biomass of <i>Bt</i> cotton stimulated the activities of all enzymes	Sun et al. (2007)
Cry1Ac	N mineralization and Olsen-P	Soil with <i>Bt</i> and non- <i>Bt</i> cotton	Total mineral-N was reduced in <i>Bt</i> cotton, whereas Olsen-P was increased	Sarkar et al. (2008)
Cry1Ac	Root biomass	Soil with <i>Bt</i> and non- <i>Bt</i> cotton	Root biomass were not different but root volume was significantly higher in <i>Bt</i> than non- <i>Bt</i> isolate	Sarkar et al. (2008)
Cry1Ac	Microbial biomass C, N and P, organic carbon, microbial quotient, potential nitrogen mineralisation, nitrification, nitrate reductase, AcP, ALP, root volume	Soil with <i>Bt</i> and non- <i>Bt</i> cotton	No negative effects of <i>Bt</i> -cotton on the indicators.	Sarkar et al. (2009)
Cry1Ac	Urease activity, nitrate reductase, Acid and alkaline phosphatases	Soil with <i>Bt</i> and non- <i>Bt</i> cotton	No significant difference enzyme activities	Mina et al. (2011)
Cry1Ac	Dehydrogenase and KMnO_4 -N content	Soil with <i>Bt</i> and non- <i>Bt</i> cotton	Positive correlations between <i>Bt</i> cotton cultivation and KMnO_4 -N content and dehydrogenase in soil	Singh et al. (2013)
Cry1Ac	Soil respiration, fluorescein diacetate hydrolysis, urease, dehydrogenase, microbial biomass carbon	Soil with <i>Bt</i> and non- <i>Bt</i> cotton	Higher biological activities in soil grown with <i>Bt</i> cotton than the non- <i>Bt</i> cotton	Velmourougane and Sahu (2013)

Table 8.7 Effects of Cry proteins from *Bacillus thuringiensis* on soil invertebrates

Protein	Organism	Experiment	Findings	References
Cry1Ab Cry1Ac	Collembola Mites	Fed leaves of <i>Bt</i> and non- <i>Bt</i> cotton	No significant effects on oviposition, nos. of eggs, and body length	Yu et al. (1997)
Cry1Ac	Earthworm	Transgenic cotton	No adverse effect on earthworms	Valasubramanian (2001)
Cry1Ab Cry1Ac	Collembola	Cultivation of <i>Bt</i> and non- <i>Bt</i> cotton	No effects on numbers	USEPA (2001)
Cry1Ac	Micro, meso and macro fauna	Cultivation of <i>Bt</i> and non- <i>Bt</i> cotton	micro, meso and macro fauna were more in <i>Bt</i> cotton rhizosphere	Mina et al. (2011)

plant species (maize, cotton, wheat, and rice), with targeted goals such as resistance to insect pests or herbicides, increased growth, and increased nutritional quality. Enormous studies have been conducted to assess the potential beneficial and/or detrimental effects of genetically engineered plants on soil microbes. Special attention was paid to study the impact of *Bt* plants, which express the cry toxin of *B. thuringiensis*, on microbial communities. Though few studies demonstrated a negative impact of *Bt* crops on soil microbes (Castaldini et al. 2005; Wu et al. 2004), most of the studies showed no adverse effects (Baumgarte and Tebbe 2005, Blackwood and Buyer 2004; Brusetti et al. 2004; Devare et al. 2004, Griffiths et al. 2005; Liu et al. 2005, Muchaonyerwa et al. 2004; Saxena and Stotzky 2001).

From the already published work, following points emanate:

1. *Bt* cotton provided effective control against lepidopteran pests and reduced insecticide spray applications.
2. Cry proteins released in root exudates and from *Bt* cotton residues appear to have no consistent, significant, and long-term effects.
3. Differences in numbers and community structure of microorganisms in soil between *Bt* and non-*Bt* crops were not statistically significant and transient.
4. Although Cry proteins bind rapidly on clays and humic substances, there is little evidence for the accumulation of the proteins in soils in the field, even after years of continuous cultivation of *Bt* cotton.

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Chapter 9

Microbial and Functional Diversity of Vermicompost Bacteria

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Abstract Vermicompost, an excellent biofertilizer, is the product of non-thermophilic biodegradation of organic material produced by the combined action of earthworms and associated microbes in the process termed as vermicomposting. Vermicompost is a finely divided peat like material with high porosity, aeration, water holding capacity, low C:N ratios, excellent nutrient status and microbial diversity and activity. The microbes associated with vermicompost promote the plant growth directly by production of plant growth-promoting (PGP) enzymes and hormones such as 1-aminocyclopropane-1-carboxylate (ACC) deaminase and indole-3-acetic acid (IAA), phosphate solubilization and siderophore production. Microbes also enhance plant growth indirectly by the suppression of plant pest and diseases. Vermicomposting technology apart from producing the biofertilizer enriched with beneficial microbes, also acts as a “panacea” for management of tons of organic wastes generated each day from urban and rural areas which if not disposed properly will have a serious impact on the environment and life on earth. Thus, vermicompost microbes help to improve soil fertility, promote plant growth and play a key role for effective management of wastes.

9.1 Introduction

The term “vermicompost” originated from a Latin word “*vermes*” meaning “worms” and the process of composting of organic material using earthworms is known as vermicomposting. Vermicompost, an excellent biofertilizer is a peat-like material produced by the joint action of earthworms and associated microbes. A wide variety of organic materials ranging from kitchen wastes to hospital bio-solids can be vermicomposted. Earthworms feed upon organic material and as this organic material passes through the earthworm’s gut they are acted upon by earthworms gut associated microbes and gets converted into finely divided peat-like material fortified with plant growth-promoting nutrients and hormones and termed as vermicompost.

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This mesophilic biological oxidation process is known as “vermicomposting.” Due to richness in nutrient availability and microbial activity vermicompost increase soil fertility, enhance plant growth and suppress the population of plant pathogens and pests. Additionally, vermicompost also improves soil physio-chemical characteristics by increasing the soil porosity, aeration, drainage and water-holding capacity, in addition to enrichment of bacterial diversity and activity.

9.2 Earthworms

There are nearly 3627 species of terrestrial earthworms in the world (Reynolds 1994). Earthworms rightly termed as “farmer’s friend” inhabit wide range of soils representing 60–80% of the total soil biomass and are indicators of soil health. Darwin narrated them “as the unheralded soldiers of mankind.” Earthworms, the “nature’s ploughman” act as “ecosystem engineers and soil conditioners” as they play a major role in altering the physio-chemical and biological properties of soil by crushing, grinding, aerating and stabilizing the soil preventing soil compaction and erosion. Further, earthworms play an essential role in carbon turnover, soil formation, participates in cellulose degradation and humus accumulation. Besides chemically detoxifying the soil aiding in bioremediation they also act as a “bio-stimulant” making the soil conducive for the enrichment of beneficial soil microbes. Earthworms are reported to promote the growth of ‘beneficial decomposer bacteria’ in wastewater and acts as aerators, grinders, crushers, chemical degraders, and biological stimulators (Dash 1978; Sinha et al. 2002) and are known to be associated with a diverse group of microbes including bacteria, fungi and actinomycetes. Based on their habitat, earthworms are classified into three main eco-physiological categories namely the ‘epigeics’ or the compost dwelling worms which include *Eisenia foetida*, *Lumbricus rubellus*, *L. castaneus*, *L. festivus*, *Eiseniella tetraedra*, *Bimastus minusculus*, *B. eiseni*, *Dendrodrilus rubidus*, *Dendrobaena veneta*, *D. octaedra*; the ‘endogeics’ or the topsoil or subsoil dwelling worms which include *Aporrectodea caliginosa*, *A. trapezoides*, *A. rosea*, *Millsonia anomala*, *Octolasion cyaneum*, *O. lacteum*, *Pontoscolex corethrurus*, *Allolobophora chlorotica*, *Aminthas* sp., and the ‘anecics’ or the worms that dwell in permanent deep burrows and they include *L. terrestris*, *L. polyphemus* and *A. longa*. Among them “epigeics” are known to be “efficient compost producers.” These voracious feeders of organic wastes utilize only a small portion of wastes by consuming for their growth and excrete a large proportion of wastes in a half-digested form which are later acted upon by earthworms gut associated microbes, enzymes and hormones which aid in rapid decomposition transforming them into vermicompost (Edwards and Lofty 1977; Kale and Bano 1986; Jambhekar 1992; Nagavallemma et al. 2004).

9.2.1 Earthworm Gut

The gut of earthworm is considered as a microbe enriching chamber. It is a straight tube starting from mouth followed by a muscular pharynx, oesophagus, thin-walled

crop, muscular gizzard, foregut, midgut, hindgut, associated digestive glands and ending with anus. The gut consisted of mucus containing protein and polysaccharides, organic and mineral matter, amino acids and microbial symbionts viz., bacteria, protozoa and microfungi with ability to digest a wide range of organic materials and polysaccharides including cellulose, sugars, chitin, lignin, starch and polylactic acids (Pathma and Sakthivel 2012). The increased organic carbon, total organic carbon and nitrogen and moisture content in the earthworm gut provide an optimal environment for the activation of dormant microbes and germination of endospores etc. Also, the microbes entering the earthworms gut feed upon the nitrogenous compounds of the mucus, which largely increase their activity, enabling them to produce enzymes such as amylase, cellulase, protease, lipase, chitinase, mannase and urease which contributes to the digestive processes of the earthworms (Pramanik et al. 2007; Munnoli et al. 2010).

9.3 Biology and Physio-chemistry of Vermicomposting

Tonnes of degradable organic wastes including crop residues, agro-industrial, dairy, kitchen and municipal wastes, hospital bio-solids and sewage sludge generated from urban and rural areas pose a major threat for safe disposal. Composting comes to rescue which converts these bulk, infectious material into less bulk, non-infectious forms which can be reused. Vermicomposting proved to be the best alternative resource recovering technology compared to conventional composting and differs from it in several ways (Gandhi et al. 1997). An array of organic wastes ranging from kitchen waste, agro-residues, municipal wastes and hospital bio-solids can be vermicomposted (Pathma and Sakthivel 2012). Vermicomposting significantly altered the physio-chemical and biological characteristics of the parent material used. Conventional composting is a thermophilic process in which microbes takes the sole responsibility for decomposition requiring a prolonged period of nearly 20 weeks for compost production (Bernai et al. 1998; Sánchez-Monedero et al. 2001) whereas vermicomposting is a mesophilic bio-oxidation process in which both earthworm and their gut associated microbes shares the responsibility for decomposition thereby aiding in rapid decomposition in a short time of 4–8 weeks (Ghosh et al. 1999; Nagavallemma et al. 2004). Apart from hastening the decomposition process by 2–5 times, vermicomposting produces much more homogenous materials compared to thermophilic composting (Bhatnagar and Palta 1996; Atiyeh et al. 2000a). NMR spectroscopic analyses showed that vermicomposting induced more lignolysis and organic matter humification of woody plant material compared to traditional composting (Vincelas-Akpa and Loquet 1997).

Earthworms comminute the substrates, thereby increases the surface area for microbial degradation constituting to the active phase of vermicomposting. As the organic matter passes through the earthworms gizzard it is ground into a fine powder which is subjected to the action of the digestive enzymes, microorganisms and other fermenting substances, further aiding their breakdown within the gut, and finally passes out in the form of “casts” which are later acted upon by earthworm gut associated microbes that takes up the process of decomposition contributing

to the maturation phase (Lazcano et al. 2008) converting them into matured final product, the “vermicompost” (Dominguez and Edwards 2004) which occurs once the worm’s moves to fresher layers of undigested waste. The duration of the active phase depends on the species and density of the earthworms involved (Ndegwa et al. 2000; Lazcano et al. 2008; Aira et al. 2011). Also, distinct differences existed between the microbial communities and thereby the microbial processes in vermicomposting and composting (Subler et al. 1998; Pathma and Sakthivel 2012). Traditional composting involves thermophilic bacterial community while vermicomposting is characterized by mesophilic bacteria and fungi (Benitez et al. 1999; Lazcano et al. 2008; Vivas et al. 2009). Actinobacteria and γ -Proteobacteria were abundant in vermicompost, while conventional compost contained more α -Proteobacteria and Bacterioidetes, the bacterial groups typical of non-cured compost (Vivas et al. 2009).

Analysis of vermicompost for the measurement of the pH, EC, organic carbon (OC) (Nardi et al. 1983; Albanell et al. 1988; Mitchell 1997), C:N ratio (Riffaldi and Levi-Minzi 1983; Albanell et al. 1988), nitrogen and potassium exhibited lower while the content of humic acids, micronutrients and total phosphorous were higher in vermicompost in comparison to that of the parent material used (Hashemimajid et al. 2004). Mineralization of N and P, microbial decomposition of organic materials into intermediate organic acids, fulvic acids, humic acids (Lazcano et al. 2008; Albanell et al. 1988; Chan and Griffiths 1988; Subler et al. 1998) and concomitant production of CO_2 (Elvira et al. 1998; Garg et al. 2006) are factors responsible for slightly decreased pH values of vermicompost compared to traditional compost. During bio-oxidation process, CO_2 and N are lost and loss of N takes place at a comparatively lower rate, hence C:N ratio indicates the degree of decomposition. As vermicompost underwent intense decomposition it had significantly low C:N ratio compared to conventional compost (Lazcano et al. 2008). Vermicomposts contain higher nutrient concentrations, but less likely to produce salinity, than composts. EC represents the salinity of the organic amendment and vermicompost contain less soluble salts and greater cation exchange capacity compared to the parent material used (Holtzclaw and Sposito 1979; Albanell et al. 1988). Vermicomposts showed a significant increase in exchangeable Ca^{2+} , Mg^{2+} and K^{+} compared to fresh sludge indicating the conversion of nutrients to plant-available forms (Garg et al. 2006; Yasir et al. 2009). Though organic matter is rich in humic acid substances, vermicomposting drastically enhances the humification processes by fragmentation and size reduction of organic matter, increased microbial activity within earthworm intestine; soil aeration by earthworm feeding and movement and thereby increases the rate of production of humic acids from 40 to 60% compared to traditional composting. (Albanell et al. 1988; Dominguez and Edwards 2004). The increase of total phosphorous (TP) in vermicompost is plausibly due to mineralization and mobilization of phosphorus resulting from the enhanced phosphatase activity by earthworms gut associated microorganisms (Zhang et al. 2000; Garg et al. 2006). The concentration of nitrate-nitrogen increased to 28-fold in vermicompost, while in conventional compost it is only 3-fold increase (Subler et al. 1998; Atiyeh et al. 2000a). Also, vermicomposting increases the ash concentration and in turn increases the rate of mineralization, the process in which the chemical compounds in the organic matter decompose or oxidise into forms that could be easily assimilated by the plants. In addition ash hinders the H_2S formation as well as improves the O_2 availability

thereby renders the composts odourless (Albanell et al. 1988; Pathma and Sakthivel 2012). Soil supplemented with vermicompost showed better plant growth compared to soil treated with inorganic fertilizers or cattle manure (Kalembasa 1996; Subler et al. 1998).

9.4 Taxonomic and Functional Diversity of Bacteria Associated with Vermicompost

Apart from improving physio-chemical properties of soil, vermicomposts also possess outstanding biological properties as they harbour significantly larger and more diverse microbial populations compared to conventional composts (Edwards 1998). Worm treated compost showed considerable increase in total viable counts of bacteria and actinobacteria (Devi et al. 2009). Interaction between earthworms and microorganisms is quite complex. Earthworms improve aeration through burrowing actions. They fragment the organic substrate thereby increases its surface area for microbial action, and in addition its gut mucus nourishes the microbes thereby stimulates and accelerates the microbial activities resulting in increasing the soil microbial population (Binet et al. 1998), microbial numbers and biomass (Edwards and Bohlen 1996). The mesophilic nature of vermicompost conserves the microbial diversity while the earthworms gut acts as conducive environment for enrichment of beneficial plant growth-promoting microbes. As the earthworm feeds upon rhizospheric soil they also ingest rhizobacteria such as *Pseudomonas*, *Rhizobium*, *Azospirillum*, *Azotobacter* *Bacillus*, etc. which get activated or increased due to the ideal micro-environment of the gut. Therefore, earthworm activity increases the population of plant growth-promoting rhizobacteria (PGPR) (Sinha et al. 2010). Bacteria belonging to specific phylogenetic groups such as fluorescent pseudomonads, actinobacteria and *Aeromonas hydrophila* were found in the vermicasts of *L. terrestris* (Devliegher and Verstraete 1997), *L. rubellus* (Kristufek et al. 1993) and *E. foetida* (Toyota and Kimura 2000) respectively in higher numbers. Singleton et al. (2003) reported the presence of 'nitrogen-fixing' and 'decomposer microbes' in earthworms gut and their casts. Suhane (2007) observed the presence of *Nitrobacter*, *Azotobacter*, *Rhizobium*, phosphate solubilizers and actinobacteria in vermicompost where the total bacterial counts exceeded 10^{-10} /g of vermicompost. Vaz-Moreira et al. (2008) evaluated vermicompost for the presence of Firmicutes viz., *Bacillus benzoovorans*, *B. cereus*, *B. pumilus*, *B. subtilis*, *B. licheniformis*, *B. megaterium*, *B. macrolides*, Actinobacteria namely *Cellulosimicrobium cellulans*, *Microbacterium* sp. *M. oxydans*, Proteobacteria such as *Pseudomonas* sp. *P. libaniensis*; ungrouped genotypes *Sphingomonas* sp., *Kocuria palustris* and yeasts namely *Geotrichum* sp. and *Williopsis californica*. Molecular and culture-dependent analyses of bacterial community identified the presence of α -Proteobacteria, β -Proteobacteria, γ -Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes and Planctomycetes in vermicompost (Yasir et al. 2009). Single-strand conformation polymorphism (SSCP) profiles the diversity of eight bacterial groups viz., α -Proteobacteria, β -Proteobacteria, γ -Proteobacteria, δ -Proteobacteria, Bacteroidetes, Verrucomicrobia, Planctomycetes, and Firmicutes from fresh soil, gut, and casts

of the earthworms *L. terrestris* and *Aporrectodea caliginosa* showed the presence of α -Proteobacteria, β -Proteobacteria, Bacteroidetes, Flavobacteria, Sphingobacteria and *Pseudomonas* sp. worm casts in addition to unclassified Sphingomonadaceae and *Alcaligenes* sp. (Nechitaylo et al. 2010). Bacteria belonging to genera *Pseudomonas*, *Bacillus*, *Microbacterium*, *Acinetobacter*, *Chryseobacterium*, *Arthrobacter*, *Pseudoxanthomonas*, *Stenotrophomonas*, *Paenibacillus*, *Rhodococcus*, *Enterobacter*, *Rheinheimera* and *Cellulomonas* were isolated from straw and goat manure based vermicompost produced by earthworm *E. foetida* (Pathma and Sakthivel 2013). Majority of them possess antagonistic and bio-fertilizing potential. Around 49% of the vermicompost bacteria isolated showed antagonism against one or more devastating phytopathogenic fungus while 22% of them showed antibacterial activity against human pathogens under in vitro conditions. Additionally 26% of the bacterial reported in this study produced indole-3-acetic acid (IAA), 51% produced siderophores, 64% produced ACC deaminase, 27% showed phosphate solubilization while 12% of the bacterial population produced hydrogen cyanide (HCN). Majority of the bacteria produced various extracellular enzymes such as cellulase, protease, lipase, xylanase, chitinase, amylase, and gelatinase (Pathma and Sakthivel 2013) (Table 9.1).

Characterization and quantification of the enzymatic activity has a direct correlation with microbial diversity and population and reflects the dynamics of the composting process in terms of the decomposition of organic matter and nitrogen transformations which in turn narrates the maturity of the compost (Tiquia 2005). Dehydrogenase, an intracellular enzyme involved in oxidative phosphorylation acts as an indicator of the microbial activity in soil and other biological ecosystems (Garcia et al. 1997). Maximum activity of cellulase, amylase, invertase, protease and urease were recorded at a short duration of 21–35 days in vermicompost and on a prolonged duration of 42–49 days in conventional composting. Additionally, microbial populations and their extracellular enzyme profiles were more abundant in vermicompost produced from cow-dung, fruit-pulp, vegetable waste and groundnut husk compared to the conventional composts produced from the same substrate (Devi et al. 2009).

9.5 Vermicomposting: A Tool for Infectious Waste Management

Disposal of sewage sludge, bio-solids and biomedical waste by safe and cheaper means is indispensable. All these wastes are infectious as they contain an array of pathogenic microorganisms and hence their disinfection is a prerequisite before being disposed into the environment (Masciandaro et al. 2000). Vermicomposting is reported to play a vital role in removal of enteric bacteria from biomedical wastes and solid wastes generated from wastewater treatment plants. Earthworm activities accelerated sludge stabilization and reduced the levels of pathogens and odours of putrefaction in sewage sludge (Brown and Mitchell 1981; Hartenstein 1983). Earthworms increase the hydraulic conductivity and natural aeration by granulating the clay particles and in addition they increase the total specific surface area by

Table 9.1 Microbial and functional diversity vermicompost bacteria

Vermicompost earthworm species	Ecological category	Vermicompost bacteria	Functional traits	References
<i>Lumbricus rubellus</i>	Epigeics	<i>Rhizobium japonicum</i> , <i>Pseudomonas putida</i>	Plant growth promotion	Madsen and Alexander (1982)
<i>L. terrestris</i>	Anecics	<i>Bradyrhizobium japonicum</i>	Improved distribution of nodules on soybean roots	Rouelle (1983)
<i>Aporrectodea trapezoids</i> , <i>A. rosea</i>	Endogeics	<i>P. corrugata</i> 214OR	Suppress <i>Gaeumannomyces graminis</i> var. <i>Tritd</i> in wheat	Doube et al. (1994)
<i>A. trapezoids</i>	Endogeics	<i>R. meliloti</i> L5–30R	Increased root nodulation and nitrogen fixation in legumes	Stephens et al. (1994)
<i>Eisenia foetida</i>	Epigeics	<i>Bacillus</i> sp., <i>B. megaterium</i> , <i>B. pumilus</i> , <i>B. subtilis</i>	Antimicrobial against <i>Enterococcus faecalis</i> DSM 2570, <i>Staphylococcus aureus</i> DSM 1104	Vaz-Moreira et al. (2008)
<i>L. terrestris</i>	Anecics	Fluorescent pseudomonads, Filamentous actinomycetes	Suppress <i>Fusarium oxysporum</i> f. sp. <i>asparagi</i> and <i>F. proliferatum</i> in asparagus, <i>Verticillium dahlia</i> in eggplant and <i>F. oxysporum</i> f. sp. <i>lycopersici</i> Race 1 in tomato	Elmer (2009)
<i>Eudrilus</i> sp.	Epigeics	Free-living N ₂ fixers, <i>Azospirillum</i> , <i>Azotobacter</i> , <i>Nitrobacter</i> , Autotrophic <i>Nitrosomonas</i> , Ammonifiers, Phosphate solubilizers, <i>Pseudomonas</i>	Plant growth promotion by nitrification, phosphate solubilisation and plant disease suppression	Gopal et al. (2009)

Table 9.1 (continued)

Vermicompost earthworm species	Ecological category	Vermicompost bacteria	Functional traits	References
<i>E. foetida</i>	Epigeics	Proteobacteria, Bacteroidetes, Actinobacteria, Verrucomicrobia, Firmicutes	Antifungal against <i>Colletotrichum coccodes</i> , <i>R. solani</i> , <i>P. ultimum</i> , <i>P. capsici</i> and <i>F. moliniforme</i>	Yasir et al. (2009)
<i>E. foetida</i>	Epigeics	Strains of <i>Pseudomonas</i> , <i>Bacilli</i> , <i>Acinetobacter</i> , <i>Microbacterium</i> , <i>Chryseobacterium</i> , <i>Arthrobacter</i> , <i>Enterobacter</i> , <i>Rheinheimera</i> , <i>Pseudoxanthomonas</i> , <i>Stenotrophomonas</i> , <i>Cellulomonas</i> and <i>Rhodococcus</i>	Production of indole-3-acetic acid, 1-amino-cyclopropane-1-carboxylate deaminase, phosphate solubilization, nitrate reduction, antagonistic against <i>Sarocladium oryzae</i> , <i>F. oxysporum</i> , <i>Pestalotia theae</i> , <i>Macrophomina phaseolina</i> , <i>Curvularia lunata</i> , <i>Colletotrichum gloeosporioides</i> , <i>Cylindrocladium floridanum</i> , <i>Cy. scoparium</i> , <i>Bipolaris oryzae</i> and human pathogenic strains of <i>B. subtilis</i> , <i>Staphylococcus aureus</i> , <i>Escherichia coli</i> , <i>Pseudomonads</i> sp., <i>Vibrio cholerae</i> , <i>Candida albicans</i>	Pathma and Sakthivel (2013)

grinding the silt and sand particles leading to enhanced adsorption of organic and inorganic matter from the wastewater. In addition, the body of earthworms acts as a 'bio-filter' and removes the biological oxygen demand (BOD), chemical oxygen demand (COD), total dissolved solids (TDS) and total suspended solids (TSS) from wastewater by 'ingestion' and biodegradation of organic wastes, heavy metals and solids by their 'absorption' through body walls (Sinha et al. 2008). Though, vermicomposting does not involve a thermophilic phase increasing the risk of its use for management of infectious waste, it surprisingly resulted in a remarkable reduction in the level of pathogen indicators such as total and faecal coliforms, *E. coli*, *Salmonella* sp., *S. enteritidis*, *Serratia marcescens*, enteric virus and helminth ova in the bio-solids (Brown and Mitchell 1981; Eastman 1999; Sidhu et al. 2001). Vermicomposting of bio-solids reduced the population of faecal coliforms and *Salmonella* sp. from 39,000 MPN/g to 0 MPN/g and <3 MPN to <1 MPN/g respectively (Dominguez and Edwards 2004). Monroy et al. (2009) reported that population of total coliforms reduced by 98% relative to that in fresh pig slurry by earthworm species *E. andrei*, *E. fetida*, and *Eudrilus eugeniae*. Similarly, vermicomposting of municipal sewage sludge using *Lampito mauritii* eliminated *Salmonella* and *Escherichia* sp. and the earthworm gut analysis also evidenced that 70 days of vermicomposting period eliminates *Salmonella* sp. ($15-17 \times 10^3$ cfu/g) and *Escherichia* sp. ($10^{-14} \times 10^2$ cfu/g) from the gut (Ganesh and Sekaran 2005). Umesh et al. (2006) found that vermicomposting replaced the infectious microbial community consisting of *P. pyocyaneae*, *Proteus vulgaris*, *S. aureus* and *E. coli* to community of commensals like *Citrobacter freundii* and aerobic spore-bearing microbes commonly inhabiting the soil and gut of earthworms. Earthworms reduces the pathogenic microbes especially the coliforms directly by feeding on them and by the action of the digestive enzymes and mechanical grinding in their gut as well as competitive interaction with earthworms native gut microflora and indirectly by promoting aerobic conditions during vermicomposting (Monroy et al. 2009; Edwards 2011; Aira et al. 2011). Recently, Pathma and Sakthivel (2013) observed that nearly 22% of the bacteria isolated from paddy straw and goat manure vermicompost showed potential antibacterial activity against human pathogens viz., *B. subtilis*, *S. aureus*, *E. coli*, *P. aeruginosa*, *V. cholerae* and *Candida albicans* under in vitro conditions which supplements that vermicomposting enriches the activity of antagonistic microbes which suppress the pathogenic inocula in infectious wastes.

9.6 Bio-fertilizing and Bio-control Potential of Vermicompost

Vermicompost is gaining importance due to its excellent nutrient status, enhanced microbial activity and antagonistic property. Vermicompost application improves soil porosity, aeration, drainage, water-holding capacity, nutrient status and microbial diversity. The rich microbial diversity of vermicompost is solely responsible

for the rich nutrient status, physiochemical properties, plant growth-promoting and bio-control properties of vermicompost.

9.6.1 Role of Vermicompost and Associated Microbes in Plant Growth Promotion

Vermicompost produced from different organic substances such as animal manure, food and kitchen wastes and crop residues enhanced the seedling growth, development and increased productivity in a variety of crops when used as a media supplement (Edwards and Burrows 1988; Atiyeh et al. 2000b). Edwards et al. (2004) reported that vermicompost application in ratio 20:1 showed a significant increase in plant growth in both field and greenhouse conditions. Addition of vermicompost to soil-less bedding plant media enhanced germination, growth, flowering and fruiting of a wide range of green-house vegetables, ornamentals such as petunias, pepper, strawberries etc. (Atiyeh et al. 2000a, b, c; Atiyeh et al. 2001; Arancon et al. 2003; Chamani et al. 2008). Aqueous extracts of vermicompost induced significant growth in petunia, begonia and coleus, comparable to that of plant growth-regulating substances (PGRs) evidencing the presence of similar substances in vermicompost (Tomati et al. 1988). Addition of earthworm casts improved plant propagation by promoting root initiation, increasing root numbers and root biomass and mushroom cultivation by stimulating carpophore formation in *Agaricus bisporus* when used as casing layer in mushroom cultivation (Tomati et al. 1987). Studies showed that biological PGP factors as auxins, gibberellins, cytokinins, ethylene and abscisic acid of microbial origin and humic acids were responsible for the fertilizing ability of vermicompost (Krishnamoorthy and Vajranabiah 1986; Tomati et al. 1988; Pathma and Sakthivel 2012, 2013). Significant amounts of (IAA), GA and cytokinins were detected in cattle manure vermicompost by HPLC and GC-MS analyses (Edwards et al. 2004). The microbial metabolites present in vermicompost were responsible for root stimulation, internode elongation and precociousness of flowering caused by vermicompost application (Tomati et al. 1987; Edwards 1998). Various bioassays using cucumber (Hahn and Bopp 1968), dwarf maize (Sembdner et al. 1976) and coleus (Edwards et al. 2004) evidenced the presence of cytokinins, GA and IAA respectively in significant quantities in vermicompost. Muscolo et al. (1996) observed that the humic substances in vermicomposts produced auxin-like cell growth and nitrate metabolism in carrots (*Daucus carota*). The humic and fulvic acids in the humus solubilize the insoluble minerals in the organic matter making them readily available to plants (Sinha et al. 2010). Humates from pig manure vermicompost and those obtained from cattle, food and paper waste vermicompost increased the growth of tomatoes, strawberries and peppers respectively (Arancon et al. 2003). Plant growth stimulating substances of microbial origin were isolated from tissues of *Aporrectodea longa*, *L. terrestris* and *Dendrobaena rubidus*. In fact, Indole like substances were detected from the tissue extracts of *A. caliginosa*, *L. rubellus* and *E. foetida* which increased the growth of peas and dry matter production of rye grass (Nielson 1965; Graff and Makeschin 1980). Supplementation

of growth media with minor quantities of vermicompost drastically increased the growth of hardy ornamentals *Cotoneaster conspicus*, *Chamaecyparis lawsonian*, *Cupressocyparis leylandi*, *Elaeagnus pungens*, *Pyracantha* sp. and *Viburnum bodnantense*. Nagavallema et al. (2004) observed that maize seedlings dipped in vermicompost water showed marked difference in plumule length compared to normal water indicating the presence of PGP hormones in vermicompost. Vermicompost application (10 t ha^{-1}) along with recommended dose of NPK increased the yield of pea (*Pisum sativum*) (Meena et al. 2007) and increased plant spread, leaf area, dry matter, fruit yield and decreased the incidence of physiological disorders namely albinism, fruit malformation and grey mould in strawberry drastically and thereby increased the marketable fruit yield. In addition the fruits possessed better quality in term of attractive coloured firmer fruits with higher total soluble solids (TSS) and ascorbic acid content. All these parameters were dose dependent and best results were achieved at 7.5 t ha^{-1} (Singh et al. 2008).

Padmavathiamma et al. (2008) observed that vermicompost amendment enhanced the nitrogen and phosphorous availability by improving biological nitrogen fixation and phosphorous solubilisation and thereby improved the biometric characters and yield in cow pea, banana and cassava grown in acidic soils. Suthar (2010) compared the effect of vermiwash and urea solution on seed germination, root and shoot length in *Cyamopsis tetragonoloba* and recorded the superiority of vermiwash to that of synthetic fertilizers. Combined application of vermicompost along with inorganic fertilizer and CIM grow® (a plant growth promoting rhizobacteria) enhanced the content of linalool, isomethane, citronellol, geraniol and citronellyl formate apart from increasing herbage and oil yield of geramium. The improved physicochemical characteristics and nutrients in plant available forms along with PGP and antagonistic disease suppressing beneficial bacteria of vermicompost might be responsible for its biofertilizing ability (Chand et al. 2011).

Pathma and Sakthivel (2013) reported that majority of vermicompost bacteria belonging to genus *Pseudomonas*, *Bacillus*, *Acinetobacter*, *Chryseobacterium*, *Arthrobacter*, *Enterobacter* and few members of *Microbacterium*, *Rheinheimera* and *Cellulomonas* were found to possess one or more PGP traits such as production of siderophores, IAA, ACC deaminase, HCN and P solubilization under in vitro conditions.

9.6.2 Control of Phytopathogens

Plants growing in soils with low organic matter are more prone to root diseases. The enrichment of this deficit soils with organic amendments can effectively suppress the disease (Raguchander et al. 1998; Lazarovits et al. 2000; Stone et al. 2004). Effectiveness of thermophilic compost has been observed in suppression of *Rhizoctonia solani* (Kuter et al. 1983), *Plasmidiophora brassicae* and *Gaeumannomyces graminis* (Pitt et al. 1998) *Phytophthora infestans* (Hoitink and Kuter 1986; Pitt et al. 1998), and *Fusarium* sp. (Kannangara et al. 2000). Addition of organic amendments to the soil improves soil health and productivity by enriching the soil beneficial microflora which apart from improving the nutrient status of the soil

by nitrogen fixation, siderophore production, phosphorous solubilisation etc., also suppresses plant diseases by microbial antagonism and competition. Since conventional composts involves a thermophilic phase, it restricts the microbial diversity while vermicomposting with its mesophilic nature encourages diversified microbial community with a majority of beneficial bacteria showing antagonism against soil phytopathogens (Chaoui et al. 2002; Scheuerell et al. 2005; Singh et al. 2008). Earthworm activity reduced the diseases caused by *Fusarium* spp., and *Verticillium dahlia* (Moody et al. 1996), *Rhizoctonia* spp. (Stephens et al. 1993) and *Gaeumannomyces* spp. (Clapperton et al. 2001). Further, *Aporrectodea trapezoides* and *A. rosea* act as vectors of *P. corrugate* 214OR, which effectively controls wheat take-all caused by *G. graminis* var. *tritici* (Doubé et al. 1994). Augmentation of pathogen infested soils in green-house with *L. terrestris* showed nearly 50–70% reduction of disease caused by *Verticillium dahliae* on eggplant (*Solanum melongena*); *Fusarium oxysporum* f. sp. *asparagi* and *F. proliferatum* on susceptible cultivars of asparagus (*Asparagus officinalis*), and *F. oxysporum* f. sp. *lycopersici* race 1 on tomato. Vermicompost incorporation in soil effectively controlled *R. solani* in wheat (Stephens et al. 1993), *Phytophthora nicotianae* and *Fusarium* in tomatoes (Nakamura 1996; Szczech and Smolinska 2001), *Plasmodiophora brassicae* in tomatoes and cabbage (Nakamura 1996), *Pythium* and *Rhizoctonia* in cucumber and radish (Simsek-Ersahin et al. 2009), *Botrytis cineria* and species of *Verticillium* in strawberry (Chaoui et al. 2002; Singh et al. 2008) and *Sphaerotheca fuliginea* in grapes (Edwards et al. 2004). Szczech et al. (1993) recorded that root dip treatment in a mixture of clay and vermicompost inhibited club-rot of cabbage caused by *P. brassicae*. Addition of vermicompost to media significantly reduced the infection by *P. nicotianae* var. *nicotianae* and *F. oxysporum* f. sp. *lycopersici* in tomato (Szczech et al. 1993) and *R. solani*, *P. drechsleri* and *F. oxysporum* in gerbera (Rodriguez et al. 2000). Potato plants treated with vermicompost were less susceptible to infection by *P. infestans* than those receiving inorganic fertilizers (Kostecka et al. 1996). Vermicompost addition to the horticulture bedding media at low rates (10–30%) effectively suppressed *Pythium* and *Rhizoctonia* under green-house conditions (Edwards et al. 2004). Under field conditions, aqueous extracts of vermicompost effectively checked powdery mildew of barley (Weltzien 1989), inhibited mycelial growth of *F. oxysporum*, *R. solani*, *Sclerotinia sclerotiorum*, *Corticium rolfsii*, and *B. cineria* (Nakasone et al. 1999). Similarly, Singh et al. (2003) observed that vermicompost extracts suppressed the development of *Erysiphe cichoracearum* on balsam (*Impatiens balsamina*) and *E. pisi* on pea (*Pisum sativum*) under field condition. Utkhedé and Koch (2004) reported that application of vermicompost tea effectively controlled bacterial canker (*Clavibacter michiganensis* sp. *michiganensis*) in tomatoes.

The disease suppressive nature of vermicompost might be attributed to microbial competition, antibiosis and hyperparasitism and systemic plant resistance (Hoitink and Grebus 1997). Chen et al. (1987) reported that a wide range of microbes were responsible for the suppression of *Pythium* and *Phytophthora* and defined this mechanism as ‘general suppression,’ while Hoitink et al. (1997) observed that the suppression of *Rhizoctonia* was brought about by a narrow range of organisms hence this mechanism was defined as ‘specific suppression.’ Experiments con-

ducted by Szczech (1999) using heat sterilized vermicompost evidenced that the disease suppressive property of vermicompost was purely biotic and Rivera et al. (2004) proved that the disease suppressive nature is dose dependent. Vermicastings are rich source of nutrients and binding agent namely calcium humate, and mucus which reduces desiccation of castings and favours the proliferation of beneficial microbes such as *Pseudomonas* sp., *Trichoderma* sp., mycorrhizal spores and chitinolytic bacterial communities viz., *Nocardioideis oleivorans*, *Streptomyces* sp. and *Staphylococcus epidermidis*. These chitinolytic bacterial communities effectively checked *R. solani*, *Colletotrichum coccodes*, *Pythium ultimum*, *P. capsici* and *Fusarium moniliforme* (Yasir et al. 2009). In vitro experiments showed that around 49% of the bacteria isolated from straw and goat manure vermicompost produced by *E. foetida* were found to be antagonistic against one or more devastating phytopathogenic fungi viz., *Sarocladium oryzae* (sheath rot of rice), *F. oxysporum* f. sp. *cubense* (banana wilt), *Bipolaris oryzae* (brown spot of rice), *Macrophomina phaseolina* (charcoal rot of groundnut), *Pestalothia theae* (leaf spot of tea), *Colletotrichum gloeosporioides* (Anthracnose of mango), *Curvularia lunata* (leaf spot of maize), *Cylindrocladium floridanum* and *C. scoparium* (root necrosis of banana) (Pathma and Sakthivel 2013). It is interesting to note that vermicompost amendment effectively reduced the incidence of ‘Yellow Vein Mosaic’, ‘Powdery Mildew’ and ‘Color Rot’ in Lady’s finger (*Abelmoschus esculentus*) (Agarwal et al. 2010). Vermicompost amendment suppressed aphids, the key vectors of plant viruses.

9.6.3 Control of Phytophagous Insects

Ramesh (2000) reported that vermicompost addition decreased the incidence of sucking pests under field conditions and the damage caused by of two-spotted *Tetranychus* sp. and *Myzus persicae* (Edwards et al. 2007) and *Pseudococcus* sp. (Arancon et al. 2007) under green-house conditions. Vermicompost addition reduced the incidence of *Heteropsylla cubana* on *Leucaena leucocephala* (Biradar et al. 1998) and *Spodoptera litura*, *Helicoverpa armigera*, *Apoaerema modicella*, *Empoasca kerri*, *Aphis craccivora* and *Tetranychus urticae* on groundnuts (Rao et al. 2001; Rao 2002, 2003). Arancon et al. (2005) reported that addition of vermicompost at a rate of less than 50% to soil less plant growth medium MetroMix 360 (MM360) reduced the damage caused by *M. persicae* and *Pseudococcus* sp. in pepper seedlings, *Pseudococcus* sp. in tomato seedlings, *M. persicae* and *Pieris brassicae* in cabbage seedlings. Vermicompost amendment reduced the infestation of *A. vittatum* and *Diabotrica undecimpunctata* on cucumbers and *Manduca quinquemaculata* on tomatoes in both green-house and field experiments (Yardim et al. 2006). Aqueous extracts of vermicompost reduced establishment and reproduction rate of *M. persicae*, *Planococcus citri*, *T. urticae* and *Acalymna vittatum* on cucumber and *Manduca sexta* on tomatoes under green-house conditions and at higher dose vermicompost teas were found to be lethal (Edwards et al. 2010a, b). George et al. (2007) observed a significant reduction in the population of *Scirtothrips dorsalis* and *Polyphagotarsonemus latus* in chilli treated with combined application

of vermicompost and vermiwash spray. Paddy fertilized with vermicompost was prone to less damage by brown plant hopper (*Nilaparvata lugens*) compared to paddy receiving equivalent NPK levels through chemical fertilizers (Bhadoria et al. 2003). Inorganic nitrogen fertilization inhibits the raise of secondary metabolite concentrations, improves the nutritional quality and palatability of the host plants (Fragoyiannis et al. 2001; Herms 2002) enhances the fecundity of insects dieting on them, attracts more individuals for oviposition (Bentz et al. 1995) and increases the population growth rates of insects (Culliney and Pimentel 1986; Jannsson and Smilowitz 1986). Vermicompost is a more stable, slower-release fertilizer giving an extended release of N, P, K, Ca, Mg and over time, thereby reducing the risk of nutrient leaching in container-based systems (Edwards and Fletcher 1988; Edwards 1998). This slow nutrient release pattern especially nitrogen helps to evade pest attack by preventing the lush green appearance of the crop which invites pests to feast upon them. In addition vermicomposts proved rich in humic acid and phenolic compounds which act as feeding deterrents thereby significantly affect pest attacks (Edwards et al. 2010a, b).

9.6.4 Control of Plant Parasitic Nematodes

Vermicomposts suppressed the damage due to plant parasitic nematodes viz., *Meloidogyne incognita* on tobacco, pepper, strawberry and tomato (Morra et al. 1998; Swathi et al. 1998; Arancon et al. 2002; Edwards et al. 2007) and decreased the numbers of galls and egg masses of *M. javanica* (Ribeiro et al. 1998). The suppression of plant parasitic nematodes by vermicompost application is possibly due to the enrichment of organic matter content of the growth media which in turn stimulates the population of microbial antagonists of nematodes (e.g., *Pasteuria penetrans*, *Pseudomonas* sp. chitinolytic bacteria, *Trichoderma* sp.), and predators of nematode including nematophagous mites viz., *Hypoaspis calcuttaensis* (Bilgrami 1996), collembola and other arthropods which selectively feeds on nematodes (Thoden et al. 2011). Earlier, Kerry (1988) reported that vermicompost amendment promoted fungi capable of trapping nematode and destroying nematode cysts while Siddiqui and Mahmood (1999) observed that addition of vermicompost increased the population of PGP rhizobacteria which produced enzymes toxic to plant parasitic nematodes. Further Wahla et al. (2012) have reported PGP pseudomonas as potential candidate for biocontrol of root-knot nematode *Meloidogyne incognita* on chilli (*Capsicum annuum*). Arancon et al. (2002) reported that tomatoes, peppers, strawberry and grapes receiving vermicomposts as nutrient supplement showed significant reduction of plant parasitic nematodes and increased the population of fungivorous and bacterivorous nematodes compared to those treated with inorganic fertilizer. Apart from the above mentioned biotic factors, vermicomposting releases hydrogen sulphide, ammonia, nitrates, and organic acids which are nematocidal in nature. Also low C/N ratio and changes in soil physiochemistry viz., bulk density, porosity, water holding capacity, pH, EC, CEC and changes in nutritional composition brought about by vermicompost application possess adverse effects on the

growth, development and fecundity of plant parasitic nematodes (Rodriguez-Kabana 1986; Thoden et al. 2011).

9.7 Concluding Remarks

Increasing awareness about the environmental issues due to indiscriminate use of synthetic fertilizers and plant protection chemicals and health benefits from organic farming has led to the use of organic manures where vermicompost finds its importance due to its cost effectiveness and improved benefits to conventional composts. Bacterial community and nutrient status of organic manures vary significantly based on the parent material and earthworm species used for vermicomposting. Hence, proper nutrient management guidelines from the scientific community would help use of vermicompost for profitable venture in addition to sustainable agriculture and earn the trust of farmers as well as motivate them to reap the benefits of eco-friendly organic agriculture.

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Chapter 10

Diversity of Cold Tolerant Phosphate Solubilizing Microorganisms from North Western Himalayas

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Abstract The environmental conditions on planet earth are extremely diverse, with enormous variations in pressure, pH, temperature and salt concentration. All these environments are inhabited by living organisms, particularly microorganisms which have adapted to the different extremes of environments. Among various extreme environments, low temperature is very common both in natural and man-made environments. Microorganisms play a significant role for maintaining the ecological balance in any ecosystem. During the long journey of evolution, they have undergone changes at different levels for adaptation and thus show huge genetic diversity for exploration. Extremely low temperature environments are generally inhabited by the cold adapted microorganisms which have the ability to grow and survive under harsh conditions. These cold adapted microorganisms, known as cold loving (psychrophiles) and cold tolerant (psychrotrophs).

The North Western Himalayan region that extends over the states of Jammu Kashmir, Himachal Pradesh and Uttarakhand in India presents a virgin opportunity for exploration and characterization of psychrophilic and psychrotolerant microorganisms, due to the unique ecological niche of the region that includes glaciers, alpine and sub alpine regions. Soil of such regions is mountaineous that is deficient in various materials. Phosphorus is second to nitrogen as a mineral nutrient required by plants and microorganisms, its major physiological role being in certain essential steps, the accumulation and release of energy during cellular metabolism. Phosphorus is an essential nutrient for plants, lack of which limits plant growth. It is least soluble in the soil. This chapter deals with the diversity of cold tolerant

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P solubilizing microorganisms from North Western Himalayas and their effect on plants with special reference to that of management of phosphorous by using cold tolerant PGPRs in improving soil quality and productivity of agricultural crops.

10.1 Introduction

Microorganisms play a major role in sustaining the productivity of an agro-ecosystem through a myriad of roles that extend from nitrogen fixation, their solubilization, nutrient mobilization to plant growth promotion and suppression of harmful pathogens and insects. A unique feature of temperate agro-ecosystems around the world is the short growing periods, which are interspersed by suboptimal temperatures. Under later conditions, most microbial processes get slow down or even come to a standstill, thereby exerting an adverse effect on the crop productivity. In the case of nutrient transformations, where microbes play a vital role this effect is most pronounced. Considering the agriculture productivity in cold environment the study of psychrotolerant (cold tolerant) microbes that retain their activity in sub-optimal temperature conditions, becomes very important. However, not many efforts had undertaken in understanding the nature and properties of such microbes. The quantum of information on the tolerance mechanisms of these agriculturally important microorganisms is also very scarce.

Phosphorus (P) is an essential element, classified as a macronutrient because plants require a relatively large amount of it. It is one of the three nutrients generally added to soils as fertilizers. One of the main roles of P in living organisms is in the transfer of energy and the P cycle, which exists in nature, explained it very well (Fig. 10.1).

Organic compounds that contain P, used to transfer energy from one reaction to drive another reaction within cells. Adequate P availability for plants stimulates early plant growth and hastens maturity. Although P is essential for plant growth, it is not freely available to them. In the process of mineralization, this is facilitated by microorganisms that convert organic phosphate present in the soil to inorganic phosphate. Therefore, the soil enriched with phosphate solubilizing microorganism is the major source of plant nutrition. There are nearly 200 mineral forms of phosphorus and the most commonly occurring form in the soil is apatite, which is an insoluble form of phosphorus.

Insoluble phosphates that are not available to the plant comprise nearly 95–99% of the total phosphates in the soil (Hayman 1975). Mineral forms of phosphorus constitute the biggest reservoirs of phosphorus, represented primarily by rocks and deposits formed during geological age. The principal characteristic of these primary minerals (oxyapatite, hydroxyapatite and apatite) is their insolubility. A major portion of soluble inorganic phosphate applied to agricultural soil as chemical fertilizer rapidly immobilized after the application and becomes unavailable to plants in long run (Dadarwal et al. 1998). Mineral phosphate also found associated with the surface of hydrated oxides of Fe, Al and Mn, which are poorly soluble and assimilable.

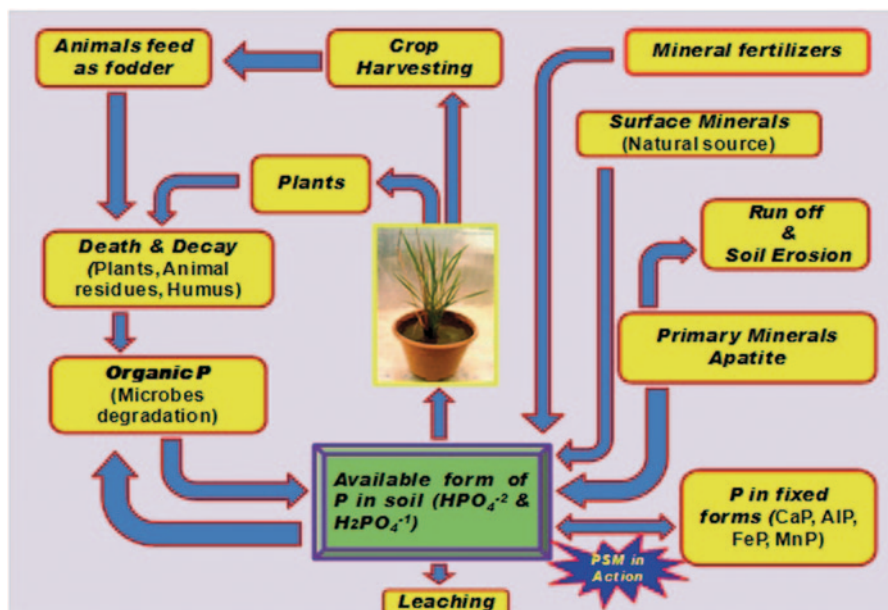


Fig. 10.1 Schematic representative of phosphorous cycle in nature with respect to plants. (Modified from Ahemad et al. (2009) In: *Phosphate Solubilizing Microbes for Crop Improvement*, Nova Science Publishers, Inc. New York.)

A second major component of soil P is organic matter, present largely in the forms of inositol phosphate (soil phytate) accounting for 50% of the total organic P. Other organic P in the soil is in the form of phosphomonoesters, phosphodiester including phospholipids and nucleic acids and phosphotriesters. Many of these P compounds are high molecular-weight material, which must first be bio-converted to either soluble ionic phosphate (Pi , HPO_4^{2-} , $\text{H}_2\text{PO}_4^{-}$) or low molecular-weight organic phosphate, to be assimilated by the cell (Goldstein 1994).

Phosphorus (P) is an important structural constituent of nucleic acids, phytin, phospholipids and plays an indispensable biochemical role in photosynthesis, respiration, energy storage and transfer, cell division, cell enlargement and several other processes in the living plant hence the application of phosphatic fertilizers become inevitable in modern agriculture (Tilak et al. 2005). Consequently, phosphorus is one of the essential plant nutrients making up 0.2% of the plant dry weight. An adequate supply of phosphorus in the early stages of plant growth promotes physiological functions including early root formation and is important for laying down the primordia for reproductive parts of plants. It is vital to seed formation and its content is higher in seeds than in any other part of the plant. It helps plants to survive winter rigors and contribute to disease resistance in some plants. Quality of many fruits, vegetables and grain crops is also improved by phosphorus. However, when P is inadequate or deficient, it reduces (i) leaf expansion, (ii) leaf surface area, (iii) number of leaves, (iv) root and shoot growth, (v) premature senescence of

leaves, (vi) delay blooming and maturity, (vii) delay flowering, (viii) reduce quality of forage, fruit, vegetable and grain crops, and (ix) decrease disease resistance. The deficiency of P in plants in general, manifested in terms of stunted growth, reduced yield and delayed maturity (Sawyer and Creswell 2000). Literature search has revealed that researches have focused on bacteria and fungi, which live in association with higher plants. While, Ratti et al. (2001) has reported that, some bacterial inoculants can enhance root colonization by mycorrhizal fungi applications. Many authors have reported a growth-promoting effect of phosphorus solubilizing micro-organisms (PSM) (Narula et al. 2000; Sundara et al. 2002).

10.2 Phosphate Solubilization by Cold Tolerant Bacteria

The bacteria, which are adapted to live in cold environmental conditions, are called as Psychrotrophs or cold tolerant bacteria. A number of such bacteria have been known from the Arctic region (Chablain et al. 1997; MacCormack and Fraile 1997). While a substantial portion of Himalaya remains frozen throughout the year, similar climatic conditions were found to prevail in alpine environments which are characterized by dramatic seasonal shift in physical and environmental condition, due to intermittent snow cover and fluctuating sub freezing temperatures in winter and intense, desiccating sunshine punctuated by infrequent rains during summer (Greenland and Losleben 2001). The observation made by Kumar et al. (2005), who were able to characterize a group of cold tolerant *Pseudomonads* from the Garhwal region of the Indian Himalaya and exhibit plant growth promotion activity at temperatures ranging from 4 to 25 °C, besides having disease-suppressing activity against major root borne diseases of garden pea. Likewise, Pandey et al. (2006) have reported the isolation and characterization of a cold tolerant phosphate solubilizing and antagonistic strain of *Pseudomonas putida* strain from sub alpine Himalayan region. The strain was able to solubilize P at cold temperatures range (4–28 °C). In a similarly type of study carried out by Selvakumar et al. (2008a, 2009c) explored the N.W. Himalayan region and reported cold tolerant phosphate solubilizing bacteria *Pantoea dispersa* and *Exiguobacterium acetylicum*. These strains solubilized phosphate at temperatures ranging from 4 to 30 °C, besides significantly increasing the percent germination, rate of germination, plant biomass and nutrient uptake of wheat seedlings under cold temperature conditions. A rhizosphere competent phosphate solubilizing strain of *Acinetobacter rhizosphaerae*, was isolated from the cold deserts of the Indian Himalayan region by Gulati et al. (2009). Another report by Selvakumar et al. (2008b) on *Serratia marcescens* strain SRM which was isolated from the flowers of summer squash plants, which was able to solubilize 76.6 µg ml⁻¹ of P and produce Indole Acetic Acid (IAA) (11.1 µg ml⁻¹). HCN and siderophore production were also detected at 15 °C. The isolate retained all the plant growth-promoting traits at 4 °C. The seed bacterization with the isolate significantly enhanced plant biomass and nutrient uptake of wheat seedlings grown at cold temperatures. The above reported study showed that phosphate solubilization by rhizospheric microflora is one of the most important means of achieving plant growth promotion.

Although, the first report of P solubilization at low temperatures was made by Das et al. (2003) who studied cold tolerant *Pseudomonas* mutants for their phosphate solubilization activity at low temperature (10 °C) and found that all the cold tolerant mutants were more efficient than their respective wild type counterparts at 10 °C as compared to 25 °C. P solubilization by *Pseudomonas* mutant's at the psychrotolerant range has also been reported by Katiyar and Goel (2003) and Trivedi and Sa (2007). However, considering the environmental stability of mutant strains, for commercial inoculant production it would be prudent to scout pristine environments for naturally occurring psychrotolerant strains. Most progress made in this direction, mainly from the Indian Himalayan Region. The studies been mentioned above are mostly of exploratory nature, while the real need of the hour is the development of commercially viable cold tolerant PSB inoculants that can be profitably used in temperate agriculture. Such microorganisms are an important component of the soil and influence the soil health through their beneficial or detrimental activities, it is imperative to study the existence of cold adapted bacterial strains with beneficial traits in the soil of the sub alpine regions for use as plant growth promoting bioinoculants.

10.3 Diversity of Cold Tolerant Phosphate Solubilizing Microorganisms

Phosphate solubilizing microorganisms (PSM) include largely bacteria and fungi, which can grow on tricalcium, iron, aluminum phosphate, hydroxyapatite, bone meal, rock phosphate and similar insoluble phosphate compounds containing media as the sole phosphate source (Table 10.1).

Such microbes not only assimilate P but a large portion of soluble phosphate is also releases in excess quantities of their own requirement (Gaur 1990). Diversified microorganism bearing ability to solubilize mineral phosphate has been studied by various researchers including Sundara and Sinha (1963); Taha et al. (1969); Barea et al. (1976); Banik and Dey (1981); Venkateshwarlu et al. (1984); Sattar and Gaur (1985); Ali et al. (1989); Gaiind and Gaur (1999); Rajarathinam et al. (1995); Bhattacharya et al. (1998); Kole and Hajra (1997, 1998). *Pseudomonas striata*, *P. cissicola*, *P. fluorescens*, *P. pinophilum*, *P. putida*, *P. syringae*, *P. aeruginosa*, *P. putrefaciens* and *P. stutzeri* have been isolated from rhizosphere of *Brassica*, chickpea, maize, soybean and other crops, desert soils and Antarctica lake (Bardiya and Gaur 1974; Jisha 1997; Nair and Rao 1977; Kole and Hajra 1997; Gupta et al. 1998; Pal et al. 2000). Various workers reported the isolation of PSB strains belonging to *Bacillus* genus such as *B. brevis*, *B. cereus*, *B. circulans*, *B. firmus*, *B. licheniformis*, *B. megaterium*, *B. mesentericus*, *B. mycoides*, *B. polymyxa*, *B. pumilis*, *B. amyloliquefaciens* and *B. subtilis* from rhizosphere of legumes, cereals (rice, maize), arecanut palm, oat, jute and chilli (Tilak 1991; Tilak et al. 2005; Kim et al. 1998; Idriss et al. 2002; Tye et al. 2002). Similarly, several *Pseudomonads* species viz., *P. putida* (Kumar et al. 2001), *P. aeruginosa* (Musarrat et al. 2000), *P. corrugata* (Pan-

Table 10.1 Diversity of important P-solubilizing microorganisms

Genera		Reference
Bacteria	Fungi	
<i>B. brevis</i> , <i>B. cereus</i> , <i>B. circulans</i> , <i>B. firmus</i> , <i>B. licheniformis</i> , <i>B. megaterium</i> , <i>B. mesentericus</i> , <i>B. mycoides</i> , <i>B. polymyxa</i> , <i>B. pumilis</i> , <i>B. pulvifaciens</i> and <i>B. subtilis</i>	<i>A. niger</i> , <i>A. flavus</i> , <i>A. nidulans</i> , <i>A. awamori</i> , <i>A. carbonum</i> , <i>A. fumigatus</i> , <i>A. terreus</i> and <i>A. wentii</i>	Taha et al. (1969); Barea et al. (1976); Banik and Day (1981); Venkateshwarlu et al. (1984); Sattar and Gaur (1985); Ali et al. (1989); Gaiind and Gaur (1999); Rajarathinam et al. (1995); Bhat-tacharya et al. (1998); Kole and Hajra (1997); Kole and Hajra (1998); Subba and Bajpai (1965); Chhonkar and Subba Rao (1967); Prerna et al. (1997)
<i>Pseudomonas striata</i> , <i>P. cissicola</i> , <i>P. fluorescens</i> , <i>P. pinophilum</i> , <i>P. putida</i> , <i>P. syringae</i> , <i>P. aeruginosa</i> , <i>P. putrefaciens</i> , <i>P. stutzeri</i> , <i>P. corrugata</i> , <i>P. fragi</i> , <i>P. lurida</i> and <i>P. poae</i>	<i>Paecilomyces fusisporus</i> , <i>Penicillium digitatum</i> , <i>P. simplicissimum</i> , <i>P. aurantiogriseum</i> , <i>Sclerotium rolfsii</i> and species of <i>Cephalosporium</i> , <i>Alternaria</i> , <i>Cylindrocladium</i> , <i>Fusarium</i> and <i>Rhizoctonia</i>	Kole and Hajra (1997); Bardiya and Gaur (1974); Nair and Rao (1977); Jisha (1997); Pal (2000); Gupta (1998); Varsha-Narsian (1994); Selvakumar et al. (2009b, Subba Rao (c, Subba Rao (2011); Gulati et al. (2008); Pandey et al. (1998)
<i>Escherichia freundii</i> , <i>E. intermedia</i> , <i>Serratia phosphaticum</i> and species of <i>Achromobacter</i> , <i>Brevibacterium</i> , <i>Corynebacterium</i> , <i>Erwinia</i> , <i>Micrococcus</i> , <i>Sarcina</i> and <i>Xanthomonas</i> , <i>Exiguobacterium acetylicum</i> , <i>Pantoea dispersa</i> and <i>Serratia marcescens</i> strain SRM	<i>Yeasts</i> , <i>Torula thermophila</i> , <i>Saccharomyces cerevisiae</i> and <i>Rhodotorula minuta</i>	Gupta (1998); Mishra (1985); Datta (1982); Selvakumar et al. (2009c, 2010, 2008a, b)

dey et al. 1998), *P. lutea* (Peix et al. 2004), *P. fluorescens* (Di Simine et al. 1998), *P. rhizosphaerae* (Peix et al. 2003), *P. stutzeri* (Vazquez et al. 2000), *P. trivialis*, *P. poae* (Gulati et al. 2008) with potent phosphate solubilizing activities have been reported across the world. In addition, *Escherichia freundii*, *E. intermedia*, *Serratia phosphaticum* and species of *Achromobacter*, *Brevibacterium*, *Corynebacterium*, *Erwinia*, *Micrococcus*, *Sarcina* and *Xanthomonas* actively solubilized insoluble phosphates. Cyanobacteria, viz. *Anabaena* sp., *Calothrix brauni*, *Nostoc* sp., *Scytonema* sp. and *Tolypothrix ceylonica* also solubilize phosphate (Gupta et al. 1998).

Among phosphate solubilizing fungi, *Aspergillus niger*, *A. flavus*, *A. nidulans*, *A. awamori*, *A. carbonum*, *A. fumigatus*, *A. terreus* and *A. wentii*, *Fomitopsis* spp. have been reported from the rhizosphere of maize, soybean, chilli, tista soils, acidic lateritic soils and compost (Subba Rao and Bajpai 1965; Chhonkar and Subba Rao 1967; Prerna et al. 1997). *Paecilomyces fusisporus*, *Penicillium digitatum*, *P. simplicissimum*, *P. aurantiogriseum*, *Sclerotium rolfsii* and species of *Cephalospo-*

rium, *Alternaria*, *Cylindrocladium*, *Fusarium* and *Rhizoctonia* are other solubilizers of insoluble phosphate. Amongst yeasts, *Torula thermophila*, *Saccharomyces cerevisiae* and *Rhodotorula minuta* can solubilize inorganic phosphate (Varsha-Narsian et al. 1994). PSM inoculants include species of *Aspergillus*, *Bacillus*, *Escherichia*, *Arthrobacter* and *Pseudomonas* (Datta et al. 1982; Mishra 1985) which can add 30–35 kg P_2O_5 ha⁻¹ (Gaur et al. 2004). Beneficial effects of inoculations with phosphorus-solubilizing microorganisms to many crop plants have been described (Subba Rao 1993; Tomar et al. 1996; Dubey 1998; Pal 1998; Richardson 2001; Rodríguez and Fraga 1999). Rhizobia are, perhaps, the most promising group of PSB because of their ability to fix nitrogen symbiotically with legumes and the capacity of some strains for solubilizing insoluble inorganic phosphate compounds Halder and Chakrabartty 1993; Halder et al. 1990). Several publications have demonstrated that phosphate-solubilizing strains of *Rhizobium* and *Bradyrhizobium* increase growth and P content of non-leguminous as well leguminous plants (Chabot et al. 1996; Antoun et al. 1998; Peix et al. 2001).

Goldstein et al. (1999) demonstrated that an efficient mineral phosphate solubilizing phenotype in Gram-negative bacteria resulted from extracellular oxidation of glucose to gluconic acid via the quinoprotein glucose dehydrogenase. A unique bacterial population isolated from the roots of *Helianthus annuus jaegeri* growing at the edge of an alkaline dry lake in the Mojave Desert in Israel showed no mineral phosphate solubilizing activity and no gluconic acid production. Addition of a concentrated solution containing material washed from the roots to these bacteria in culture however resulted in production of high levels of gluconic acid. This suggested that signaling between bacteria and plant root regulated expression of direct oxidation pathway in this bacterium. The resultant acidification of the rhizosphere played a key role in nutrient availability and/or other ecophysiological parameters essential for the survival of this desert plant. The establishment and performance of PSM affected severely under stressed conditions such as high salt, pH and temperature prevalent in degraded ecosystems represented by alkaline soils with tendency to fix phosphorus (Gand and Gaur 1991).

In a screening of 4800 bacterial isolates from the root-free soil, rhizosphere and rhizoplane of *P. juliflora* growing in alkaline soils, 857 morphotypes solubilized phosphate in agar. The incidence of PSB was highest in the rhizoplane, followed by rhizosphere and root-free soil. Phosphate solubilizing ability of strain NBRI4 was higher than control in the presence of salts (NaCl, $CaCl_2$ and KCl) at 30 °C and further increased at 37 °C, while strain NBRI2601 isolated from the rhizosphere of chickpea and alkaline soils could solubilize phosphorus in presence of 10% salt, pH 12 and at 45 °C suggesting that extensive diversity search in appropriate habitats may lead to recovery of effective bacteria (Nautiyal et al. 2000). The mechanism of osmotic stress adaptation in *P. aeruginosa* PAO1 was investigated by D'Souza-Ault et al. (1993). By using natural abundance nuclear magnetic resonance spectroscopy, osmotically stressed culture was found to accumulate glutamate, trehalose, and *N*-acetylglutaminylglutamine amide, an unusual dipeptide previously reported only in osmotically stressed *Rhizobium meliloti* and *P. fluorescens*. The intracellular levels of these osmolytes were dependent on the chemical composition and the osmolal-

ity of the growth medium. It was demonstrated that glycine betaine, a powerful osmotic stress protectant, participated in osmoregulation in this organism.

Naik et al. (2008) reported a high degree of functional and genetic diversity among the phosphate solubilizing fluorescent pseudomonad bacteria. Due to their innate potential of producing an array of plant growth promoting enzymes, hormones and antifungal metabolites of these phosphate-solubilizing strains are to be in consideration for play a vital role in plant growth promotion, disease suppression and subsequent enhancement of yield.

Generally, it is widely perceived that extremity of the environmental conditions of a niche, the lower the diversity of organisms. However, most cold inhospitable environments dominated by a variety of microorganisms, thereby making them the most versatile of all life forms. The lowest temperature limit for life seems to be around -20°C , which is the value reported for bacteria living in permafrost soil and in sea ice. Microbial activity at such temperatures is restricted to small amounts of unfrozen water inside the permafrost soil or in the ice and brine channels. These contain high concentrations of salts, exopolymeric substances and/or particulate matter and fluid flow, which are maintained by concentration and temperature gradients (D'Amico et al. 2006). Cold-tolerant microorganisms are also widely harbors in refrigerated environments and have major cause of concern in the food processing and storage industry. It is not uncommon to come across a wide variety of cold tolerant microorganisms in the alpine and sub-alpine landscapes. Bacteria, archaea and eukaryotes like yeast occur in cold environments. While bacteria dominate and are present in greater diversity than archaea in polar environments, archaea are widely spread in cold, deep ocean waters (Karner et al. 2001; Deming 2002). Morphological types encountered in cold environments include spore-formers, non-spore formers and filamentous bacteria. Together they cover a wide range of metabolic types ranging from aerobes to anaerobes, and include both heterotrophs and autotrophs.

Study on phosphate solubilizing efficiency of cold tolerant bacteria and fungi have got attention in recent years (Pandey et al. 2002, 2008; Das et al. 2003; Gulati et al. 2008; and Pandey 2010b). Pandey et al. (2006) isolated and characterized a phosphate solubilizing and antagonistic bacterial strain, designated as B0, from a sub-alpine Himalayan forest site. The isolate was Gram-negative, rod shaped, $0.8\text{--}1.6\text{ }\mu\text{m}$ in size, and psychrotrophic in nature that could grow from 0 to 35°C (optimum temp. 25°C). It exhibited tolerance to a wide pH range ($3\text{--}12$; optimum 8.0) and salt concentration up to 4% (w/v). Although, it was sensitive to kanamycin, gentamicin, and streptomycin ($<10\text{ }\mu\text{g ml}^{-1}$), it showed resistance to higher concentrations of ampicillin, penicillin, and carbenicillin ($>1000\text{ }\mu\text{g ml}^{-1}$). The isolate showed maximum similarity with *P. putida* based on 16S rRNA analysis. It solubilised tricalcium phosphate under in vitro conditions. The phosphate solubilization was estimated along a temperature range ($4\text{--}28^{\circ}\text{C}$), and maximum activity ($247\text{ }\mu\text{g ml}^{-1}$) was recorded at 21°C after 15 days of incubation. The phosphate solubilizing activity coincided with a concomitant decrease in pH of the medium. The isolate also exhibited antifungal activity against phytopathogenic fungi in Petri-dish assays and produced chitinase, β -1,3-glucanase, salicylic acid, siderophore, and hydrogen cyanide. The PGP and antifungal properties were demonstrated through

a maize-based bioassay under greenhouse conditions. Although the bacterial inoculation was found to result in significant increment in plant biomass, it stimulated bacterial and suppressed fungal counts in the rhizosphere. The present studies important in respect of enumerating microbial diversity of the colder regions as well as understanding the potential biotechnological applications of native microbes. Trivedi and Pandey (2007) screened 450 bacterial isolates from a culture collection of high altitude bacteria isolated from different regions of Indian Himalaya for their low temperature phosphate solubilization activity at 4 and 10 °C using plate based assay on Pikovskaya (PVK) agar.

Gulati et al. (2008) isolated and characterized fluorescent *Pseudomonas* with high phosphate-solubilizing ability from the alkaline and calcium-rich soils with low P availability in the cold desert region of Lahaul and Spiti in the trans-Himalayas of India. Of 216 phosphate-solubilizing isolates, 12 exhibiting high solubilization of tricalcium phosphate (TCP) in NBRIP liquid culture were identified as *Pseudomonas trivialis*, *P. poae*, *P. fluorescens*, and *Pseudomonas* spp. on the basis of phenotypic features, whole-cell fatty acids methyl ester (FAME) profiles, and 16S rDNA sequencing. These isolates also showed relatively high solubilization of North Carolina rock phosphate (NCRP) in comparison to the solubilization of Mussoorie rock phosphate (MRP) and Udaipur rock phosphate (URP). The solubilization of phosphate substrates by *P. trivialis* and *P. poae* is reported for the first time. Gulati et al (2009) reported a phosphate-solubilizing bacterium *Acinetobacter rhizosphaerae* strain BIHB723 isolated from the rhizosphere of *Hippophae rhamnoides*. The strain exhibited the plant growth-promoting attributes of inorganic and organic phosphate solubilization, auxin production, 1-aminocyclopropane-1-carboxylate deaminase activity, ammonia generation, and siderophore production. A significant increase in the growth of pea, chickpea, maize, and barley was recorded for inoculations under controlled conditions. Field testings with the pea also showed a significant increment in plant growth and yield. The rifampicin mutant of the bacterial strain effectively colonized the pea rhizosphere without adversely affecting the resident microbial populations.

In a recent study, Vyas et al. (2009) screened 19 efficient phosphate-solubilizing fluorescent *Pseudomonas* isolates from the cold deserts of the trans-Himalayas, for tolerance against temperature, alkalinity, salinity, calcium salts and desiccation induced stresses. Phylogenetic analysis based on 16S rRNA gene sequencing placed these bacteria under three groups with fourteen strains in Group I including *Pseudomonas trivialis* and *P. poae*, two strains in Group II together with *Pseudomonas kilonensis* and *P. corrugata* and three strains in Group III along with *Pseudomonas jessenii* and *P. moraviensis*. Similarly, Selvakumar et al. (2009a) reported that fourteen cold tolerant phosphate solubilizing bacteria isolated from high altitude representative locations of the two major mountain aspects of the Uttarakhand Himalayas (cooler north and warmer south facing slopes) were studied. The tricalcium phosphate (TCP) solubilizing abilities of the isolates were estimated at three different incubation temperatures viz., 4, 15 and 30 °C under in-vitro conditions (Fig. 10.2a, b).

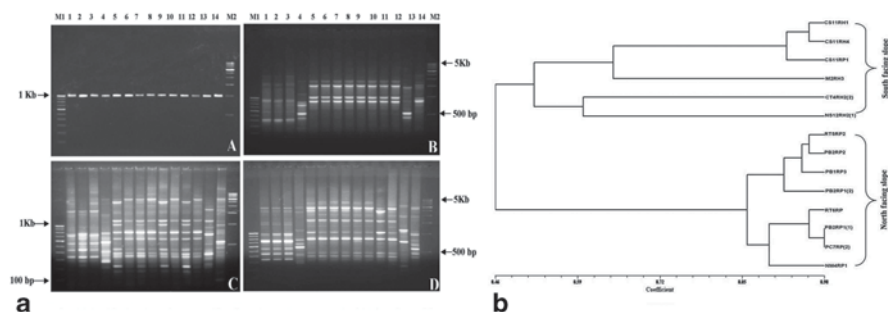


Fig. 10.2 **a** Diversity among cold tolerant *P* solubilizing bacteria from North Western Himalayas. 1 Amplification of *Pseudomonas* specific 16S rRNA gene (size approx 990 bp), 2 BOX PCR 3 ERIC PCR and 4 ERIC-2PCR amplification profile of the cold tolerant *Pseudomonads*. **b** Composite dendrogram showing the genetic relatedness between the isolates originated from different mountain North and South facing sites. (M1–100 bp ladder, M2–500 bp ladder, 1-CS11RH1, 2-CS11RP1, 3-CS11RH4, 4-CT4RH2(2), 5-RT5RP(2), 6-RT6RP, 7-PB2RP2, 8-PB2RP1(1), 9-PB1RP3, 10-PC7RP(2), 11-PB2RP1(2), 12-NM4RP1, 13- NS12RH2(1), 14-M2RH3)

Irrespective of their geographical origin, all the isolates recorded maximum *P* release values at 30 °C. At 4 °C, the isolates from the north-facing slope found to release significantly higher levels of *P*, as compared to the isolates from the south facing slopes. Alternatively, at 15 °C, the isolates from the south facing slope found to release significantly higher levels of *P*. Initial confirmation of their genus level identity as *Pseudomonads* was arrived by amplification of a 990 bp fragment of the 16S rRNA gene using genus specific primers. Further putative species level identification was arrived by sequencing of the 16S rRNA gene. The diversity among the isolates determined by rep-PCR using the primers BOX, ERIC and ERIC2. A composite dendrogram constructed using the rep-PCR profiles revealed that the isolates from the north and south mountain aspects formed separate major clusters. The extent of diversity was greater among the isolates from the south mountain aspect. The later study reveals the potential of rep-PCR in determining the genetic diversity among *Pseudomonads* selected for a single functional trait, but varying in their geographical origin.

Rinu and Pandey (2010a) reported a psychrotolerant phosphate solubilizing fungus from the rock soil of a cold desert site in Indian Himalaya. The fungus grows from 4 °C to 35 °C (optimum 21 °C), at 2 to 13.5 pH (optimum 9) under laboratory conditions. Based on phenotypic characters and 26S rDNA analysis, the fungus identified as *Paecilomyces hepiali*. In quantitative estimation that was carried out at 9, 14, and 21 °C, the fungus solubilized maximum phosphate at 14 °C. In view of the slow growth and persistence of the desired activity at low temperature, the estimation was carried out for a longer period, i.e., up to 6 weeks. The acid phosphatase activity was found to be more prominent than alkaline phosphatase in culture filtrate. High performance thin layer chromatography (HPTLC) analysis showed production of six organic acids, gluconic and α -keto glutaric acid being in maximum amount in the culture filtrate. The study has ecological significance in view of the nutrient cycling under low temperature environment, prevalent in Himalayan region.

10.4 Phosphate Solubilization by Microorganisms

Microorganisms have the potential to solubilize phosphate present in the fixed form to soluble form which is utilized by the plants. Worldwide a lot of research has been carried out on phosphate solubilizing microorganism and their role in mineralization of organic P form to inorganic form, which is easily available to the plants, is also investigated. The most efficient phosphorus solubilizing microbes belong to genera *Pseudomonas* and *Bacillus* among bacteria and *Aspergillus* and *Penicillium* among fungi. Pikovskaya (1948) demonstrated the phenomenon of P solubilization by microbes for the first time. Thereafter various other workers proved this fact by using a variety of plants, microorganisms and soils of different compositions. Various workers explaining the mechanism of microbial P solubilization in liquid medium (Kucey 1983; Asea et al. 1988; Cunningham and Kuiack 1992; Illmer and Schinner 1995). The important theories are the sink theory (Halvorson et al. 1990), the organic acid theory (Cunningham and Kuiack 1992) and the acidification by H^+ excretion theory (Illmer and Schinner 1995). According to the sink theory, P solubilizing organisms are able to remove and assimilate P from the liquid medium and therefore, stimulate the indirect dissolution of Ca-P compounds. Illmer and Schinner (1995) demonstrated that P content in the biomass of two P solubilizing organisms (*Pseudomonas* sp. and *P. aurantiogriseum*) were the same as that in non-P solubilizing. They further showed that only about 1 % of total P was actually absorbed by organisms from the solubilized phosphate present in the broth. The organic acid theory for microbial phosphate solubilization recognized and accepted by many researchers. In this theory, insoluble sources of inorganic P in liquid broth are solubilized by PSM either by lowering the pH or by enhancing chelation of the cations bound to P.

Methodologies have been developed for the rapid and efficient screening of P solubilizing bacterial cultures. Pikovskaya (1948) devised a simple plate assay system for identification of phosphate solubilizers in which phosphate solubilizers identified by the presence of clear halos around their colonies. The clear halos are formed because of the decomposition of tricalcium phosphate present in the medium in the vicinity of phosphate solubilizers due to the production of acids. Various workers for identification of phosphate solubilizers (Gupta et al. 1994; Mehta and Nautiyal 2001) have proposed other plate assay methods. The protocol developed by Mehta and Nautiyal (2001) is noteworthy as being a rapid protocol based on the decolorizing of the dye bromophenol blue for qualitative screening of the bacterial cultures for their P solubilizing ability. They included insoluble mineral phosphates such as tricalcium phosphate or hydroxyapatite as phosphate source in media. Phosphate solubilizers produce acids during their growth, which lowers the pH around their colonies causing the local decolorization of dye and thus rendering them identifiable by the presence of yellow halos around them. This method is more reproducible and has greater correlation to the phosphate solubilizing properties of the microbes in comparison to the simple halo method. Quantitative methods for measuring phosphate-solubilizing activity of bacteria in liquid medium have been demonstrated by the same workers as described above. The same medium and dye

system in liquid culture were used and spectroscopic observations gave the quantifiable output in terms of phosphate solubilization.

Pseudomonas striata and *Bacillus polymyxa* were able to solubilize 156 and 116 mg PI^{-1} respectively, under in-vitro conditions (Rodríguez and Fraga 1999). Similarly *Pseudomonas fluorescens* solubilized 100, 92 and 51 mg PI^{-1} from a medium containing $\text{Ca}_3(\text{PO}_4)_2$, AlPO_4 and Fe_3PO_4 , respectively (Henri et al. 2008). The same bacterial species was reported to solubilize tri calcium phosphate in liquid medium (29 to 105 $\mu\text{g ml}^{-1}$) on 10 days of inoculation with a simultaneous drop in pH (Naik et al. 2008). Phosphate solubilization by different bacterial strains has been reported upto a value of 200 to 805 $\mu\text{g ml}^{-1}$ of solubilized phosphate (Nautiyal and Dion 1990). *P. fluorescens* strain NJ-101 which was isolated from agricultural soil was reported to release 74.6 $\mu\text{g ml}^{-1}$ soluble phosphate from inorganic phosphate (Bano and Musarrat 2004). The mutants of the same bacterial species (CPRF8 and CRM) reported to solubilize tricalcium phosphate upto 255.2 and 23.99 $\mu\text{g ml}^{-1}$ of P at 25 °C (Katiyar and Goel 2003). Likewise, various methods have been adopted by several researchers to solubilize the insoluble phosphates and increase its availability by biologically mediated processes such as mineralization and immobilization by phosphate solubilizing organisms (Leyval and Berthelin 1989; Salih et al. 1989; Zou et al. 1992; Rodríguez and Fraga 1999). Although there is a wide range of temperatures in which phosphate-solubilizing efficiency of microbes has been reported. For example, many workers suggested that 20–25 °C is the optimum temperature at which maximum solubilization occurs (Sayer and Gadd 1998), while other researches such as Kang et al. (2002) and Varsha (2002) reported 28 °C as optimum temperature for P-solubilization. Similarly, the other authors have shown that 30 °C is the best temperature for P-solubilization (Kim et al. 1997; Rosado et al. 1998; Johri et al. 1999; Fasim et al. 2002) while solubilization at extreme temperature (45 °C in desert soil) is also reported by Nautiyal et al. (2000) and Nahas (1996). In contrast, with the above-mentioned studies, Johri et al. (1999) have also reported the phosphate solubilization by microbes at low temperature (10 °C). Similar study was carried out by Katiyar and Goel (2003) demonstrated P-solubilizing ability with subsequent effect on plant growth promotion under in-vitro and *in-situ* conditions using cold tolerant mutants of *P. fluorescens*, which grows well at 10 °C.

10.5 Mechanisms of Phosphate Solubilization

Phosphorus is one of the most important macronutrients with regard to plant growth and development. However, phosphorus available in soil readily converted into insoluble complexes, including tri-calcium-phosphate (Altomare et al. 1999). Rhizobacteria producing organic acids solubilized insoluble phosphate. The principal mechanism for inorganic mineral phosphate solubilization is the production of organic acids. In the mineralization of organic phosphorus in soil, phosphatase and phytase play a major role. The major mechanism of mineral phosphate solubilization is the action of organic acids synthesized by soil microorganisms is generally accepted. Production of organic acids results in acidification of the microbial cell

and its surroundings due to which PSB strains exhibit vast range of P-solubilizing abilities (Maheshwari et al. 2013). Tao et al. (2008) reported inorganic P-solubilizing ($25\text{--}42\ \mu\text{g P ml}^{-1}$) and organic P mineralizing ($8\text{--}18\ \mu\text{g P ml}^{-1}$). Other mechanisms, such as the production of chelating substances by microorganisms (Duff and Webley 1959) as well as the production of inorganic acids, such as sulfuric, nitric and carbonic acid has to be considered. However, their contribution to P release in soil appears to be negligible and the effectiveness of such processes has been questioned (Vázquez 1996). Over 100 years, workers have recognized the ability of soil microorganisms to solubilize P_i from insoluble (i.e., nutritionally unavailable) organic and mineral phosphates (Whitelaw 2000). Mineral phosphate solubilization by bacteria was predominantly reported at mesophilic temperatures (Chung et al. 2005; Chen et al. 2006), and has been mainly attributed to the activity of the organic acids and enzyme glucose dehydrogenase. However, recent studies have shifted the focus from P solubilization at the mesophilic range to the psychrotolerant range by mutant strains (Katiyar and Goel 2003; Trivedi and Sa 2007). Microorganisms that dissolve poorly soluble CaPs are termed as ‘mineral phosphate solubilizers’ (MPS) (Dobbelaere et al. 2003; Goldstein et al. 2003). Phosphate solubilizing microorganisms (PSMs) convert these insoluble phosphates into soluble forms through the process of acidification, chelation, exchange reactions and production of gluconic acid (Rodríguez et al. 2004; Chung et al. 2005).

10.5.1 Organic Phosphate Solubilization

Soil contains a wide range of organic substrates, which can be a source of P for plant growth. To make available this form of P for plant nutrition, it must be hydrolyzed to inorganic P. Phosphorus is released from organic compounds in soil by three groups of enzymes (i) nonspecific phosphatases, which perform dephosphorylation of phosphor-ester or phosphor-anhydride bonds in organic matter, (ii) phytases, which specifically cause P release from phytic acid and (iii) phosphonates and C–P lyases enzymes that perform C–P cleavage in organophosphonates (Fig. 10.3).

The main activity apparently corresponds to the work of acid phosphatases and phytases because of the predominant presence of their substrates in soil. Availability of organic phosphate compounds for plant nutrition could be a limitation in some soils resulting from precipitation with soil particle ions.

The capability of enzymes to perform the desired function in the rhizosphere is a crucial aspect for their effectiveness in plant nutrition. Phosphatase and phytase enzymes are carrying out mineralizations of most organic phosphorous compounds. The presence of a significant amount of phosphatase activity in soil has been reported (Kremer 1994; Sarapatka and Kraskova 1997). In different types of soils, an important level of microbial phosphatase activity has been detected (Kirchner et al. 1993; Kucharski et al. 1996). In fact, the major source of phosphatase activity in soil considered to be of microbial origin (Garcia et al. 1992; Xu and Johnson 1995) and substantially increases in the rhizosphere (Tarafdar and Junk 1987). Thus, acid phosphatases play a major role in this process. In the rhizosphere

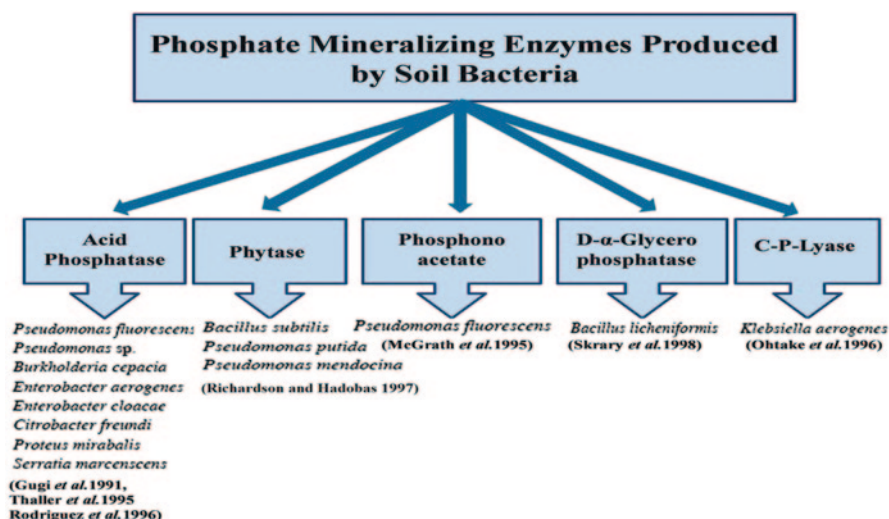


Fig. 10.3 Organic phosphate mineralizing enzymes produced by phosphate solubilizing microorganisms. (Modified from H. Rodriguez, R. Fraga/Biotechnology Advances 17 (1999))

of slash pine (*Pinus ellioti*) significant acid phosphatase activity was observed in two forest Spodosols (Fox and Comerford 1992). Earlier, Burns (1983) studied the activity of various phosphatases in the rhizosphere of maize (*Zea mays*), barley (*Hordeum vulgare*) and wheat (*Triticum aestivum*) and reported that phosphatase activity was higher in the inner rhizosphere at acidic and neutral soil pH. Soil bacteria expressing a significant level of acid phosphatases included strains from the genera *Rhizobium* (Abd-Alla 1994), *Enterobacter*, *Serratia*, *Citrobacter*, *Proteus* and *Klebsiella* (Thaller et al. 1995), *Pseudomonas* (Gügi et al. 1991) and *Bacillus* (Skrary and Cameron 1998). According to Greaves and Webley (1965), approximately 30–48% of culturable soil and rhizosphere microorganisms utilize phytate. On the other hand, Richardson and Hadobas (1997) reported that 63% of culturable soil bacteria were able to grow on this substrate as carbon and P source on agar medium. However, of these, only 39–44% could utilize phytate as a P source in liquid medium while a very low proportion could use it as a C source. All of these studies provide evidence supporting the role of bacteria in rendering organic P available to plants (Tarafdar and Claassen 1988). The degradability of organic phosphorous compounds depends mainly on the physico-chemical and biochemical properties of their molecules. For instance, nucleic acids, phospholipids, and sugar phosphates degraded easily, while phytic acid, polyphosphates, and phosphonates are decomposed slowly (Ohtake et al. 1996; McGrath et al. 1998).

Phytate is the primary source of inositol in its basic form and the major stored form of phosphate in plant seeds and pollen. Phytase activity is widespread and about 30–50% of the bacterial isolates from soil synthesizes this enzyme. Its activity in nature is enhance by addition of carbonaceous materials that increase the size of community. Species of *Aspergillus*, *Rhizopus*, *Cunninghamella*, *Arthrobacter*,

Streptomyces, *Pseudomonas* and *Bacillus* are known to synthesize these enzymes. Phytate is the major component of organic forms of P in soil (Richardson 1994). The ability of plants to obtain P directly from phytate is very limited. However, the growth and P nutrition of *Arabidopsis* plants supplied with phytate was improved significantly when they were genetically transformed with the phytase gene (*phyA*) from *Aspergillus niger* (Richardson et al. 2001b). It has been reported that phosphate mineralization of fluorescent pseudomonads from the substrates has been mediated by the enzymes, acid phosphatase and phosphonoacetate hydrolase of *P. fluorescens* strains and phytase enzyme of *P. putida*. Degradation of insoluble phytate to soluble phosphorus by microbial phytases produced by *Bacillus*, *Pseudomonas*, *Klebsiella* and *Enterobacter* sp. is reported by Greiner et al. (1997), Kerovuo et al. (1998) and Richardson et al. (2001a). The dephosphorylating reactions involve hydrolysis of phosphoester or phosphoanhydride bonds. The acid phosphohydrolases, unlike alkaline phosphatases, show optimal catalytic activity at acidic to neutral pH values. Moreover, they can be further classified as specific or nonspecific acid phosphatases, in relation to their substrate specificity. Rossolini et al. (1998) has published a comprehensive review of bacterial nonspecific acid phosphohydrolases. The specific phosphohydrolases with different activities include 3'-nucleotidases and 5'-nucleotidases (Burns and Beacham 1986), hexose phosphatases (Pradel and Boquet 1988) and phytases. A specific group of P releasing enzymes is those able to cleave C-P bonds from organophosphonates (Ohtake et al. 1996; McGrath et al. 1995).

The phosphatases production is regulated by inorganic phosphate (Pi) concentration (i.e., phosphate-repressible phosphatases) and has been best studied in the alkaline phosphatase (gene *phoA*) of *E. coli*, which is suddenly and fully induced when the Pi concentration decreases from 100 mM to 0.16 mM (Rosenberg 1987). Another Pi repressible bacterial phosphatase is the alkaline phosphatase of *Morganella morganii*, produced under low Pi availability conditions which, according to its regulation and the polypeptide components molecular mass is probably similar to that of *E. coli* (Thaller et al. 1994). *P. fluorescens* MF3, *Providencia stuartii* and *Providencia rettgeri* also produce alkaline phosphatase activity, repressed by phosphate (Gügi et al. 1991; Thaller et al. 1995). According to Kier et al. (1977), the production of the PhoN enzyme (class A acid phosphatase) of *Salmonella enterica* serovar *typhimurium* is moderately induced by Pi starvation. However, evidence has also shown that this gene is under the control of the *phoP-phoQ* two-component regulatory system (Kier et al. 1979; Miller et al. 1989), which promotes transcription of *phoN* and other *PhoP* activated genes under low environmental Mg^{2+} concentrations (Vescovi et al. 1996). The findings indicate that most of alkaline phosphatases found in the family Enterobacteriaceae are Pi-repressible, while many of the acid phosphatases are Pi-irrepressible. Other regulatory systems proposed for some bacterial phosphatases. In *P. fluorescens* MF3, it was determined that the expression of the *apo* gene, which encodes an acidic phosphatase enzyme, regulated by the growth temperature. The finding of a co-regulation mechanism at the transcriptional level suggests the existence of a new regulatory mechanism for these genes (whose expression is maximal at 17.5 °C) as a response to the growth temperature (Burini et al. 1994). Furthermore, the *apy* gene of *Shigella flexneri*,

encoding an ATP diphosphohydrolase or apyrase and other related alleles present in virulent *Shigella* spp. and enteroinvasive *E. coli* strains, is expressed in a thermo-regulated manner (Bhargava et al. 1995). Rossolini et al. (1994) reported that in *E. coli* MG1655 the production of the p27 enzyme (acid phosphatase) class B, probably corresponding to the product of the nap A gene found in this species appears to be switched off when cells were grown on glucose and turned on when growth was supported by alternative carbon sources (Thaller et al. 1995). This behavior suggests that expression of the p27 enzyme regulated in a complex fashion. All of the available evidence indicates that the regulation of phosphatase enzymes is a complex matter that requires considerable additional research. The term P solubilization by microbes seems to have undergone a paradigm shift, since recent literature on P solubilization also includes the release of organic sources of P by the action of phosphatase and phytase enzymes (Rodríguez et al. 2006).

10.5.2 Inorganic Phosphate Solubilization

It is generally accepted that the major mechanism of mineral phosphate solubilization (MPS) is through the action of organic acids produced by soil microorganisms (Leyval and Berthelin 1989; Salih et al. 1989, Rodríguez and Fraga 1999). Various organic acids identified by the liquid cultures of PSM and can be associated with specific microbial groups, e.g., 2-keto gluconic acid and oxalic acid are commonly found in bacterial and fungal cultures, respectively. Gluconic, acetic and lactic acids have been observed from both types of microorganisms and gluconic acid seems to be the principal organic acid frequently found among PSM. Among the organic acids, gluconic acid seems to be the most frequent agent of mps and is reported as the principal organic acid produced by phosphate-solubilizing bacteria, such as *Pseudomonas* sp. (Illmer and Schinner 1992; Gulati et al. 2009), *Erwinia herbicola* (Liu et al. 1992), *Pseudomonas cepacia* (Goldstein et al. 1993) and *Burkholderia cepacia* CC-A174 (Lin et al. 2006). Other organic acid identified in strains with phosphate-solubilizing ability is 2-keto gluconic acid, which is present in *R. leguminosarum* (Halder et al. 1990), *R. meliloti* (Halder and Chakrabartty 1993) and *B. firmus* (Banik and Dey 1982). Strains of *B. liqueniformis* and *B. amyloliquefaciens* found to produce mixtures of lactic, isovaleric, isobutyric, and acetic acids (Rodríguez and Fraga 1999). Henri et al. (2008) isolated three *P. fluorescens* strains [CB501, CD511 and CE509] from acidic soils of Cameroon, having the ability to solubilize the three phosphate types [$\text{Ca}_3(\text{PO}_4)_2$, $\text{AlPO}_4 \cdot \text{H}_2\text{O}$ or $\text{FePO}_4 \cdot 2\text{H}_2\text{O}$], by the production of organic acids (Fig. 10.4; Table 10.2).

Production of organic acids results in acidification of the microbial cell and its surroundings. Consequently, P_i released from a mineral phosphate by proton substitution for Ca^{2+} (Goldstein 1994). Gluconic acid seems to be the most frequent agent of mps among the organic acids and reports as the principal organic acid produced by PSB such as *Pseudomonas* sp. (Illmer and Schinner 1992), *E. herbicola* (Liu et al. 1992) and *P. cepacia* (Goldstein et al. 1993). Gluconic acid is the principal organic acid produced by *Pseudomonas* spp., *E. herbicola*, *Bacillus* spp., *Burkhold-*

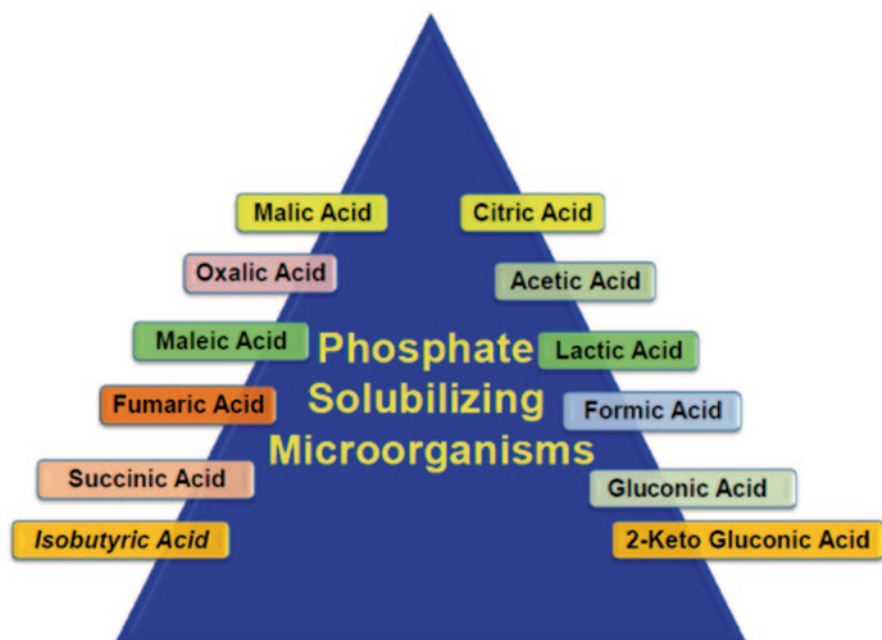


Fig. 10.4 Types of organic acids produced by various microbes during phosphate solubilization

eria spp., *Rhizobium* spp. 2-keto gluconic acid is another organic acid identified in strains with phosphate solubilizing ability. Strains of *Bacillus* found to produce mixture of lactic, isovaleric, isobutyric and acetic acids. Other organic acids, such as glycolic, oxalic, malonic, and succinic acid also identified among phosphate solubilizers. Strains from the genera *Pseudomonas*, *Bacillus* and *Rhizobium* are among the most powerful phosphate solubilizers (Rodríguez and Fraga 1999) (Table 10.3).

Due to lack of a linear correlation between pH and the amount of solubilized P, alternative possibilities for mps have been proposed (Asea et al. 1988; Ehrlich 1990). In addition, no significant amounts of organic acid production detected from a phosphate-solubilizer fungus *Penicillium* sp. Further studies have shown that the release of H^+ to the outer surface in exchange for cation uptake or with the help of H^+ translocation ATPase could constitute alternative ways for solubilization of mineral phosphates. Illmer and Schinner (1992) found that the culture filtrate of PSB's contained a number of organic acids including citric, fumaric, malic, 2-keto gluconic, gluconic and glyoxylic acids. Kpomblekou and Tabatabai (1994) postulated that the decrease in the pH of the medium is related to the production of organic acids by the PSB's and subsequent plant growth promotion by the solubilization of insoluble sources of phosphorous. Nautiyal et al. (2000) observed alkaline stress induced P solubilization by bacterial cultures under in-vitro conditions while no correlation was observed between the pH of the medium and the extent of P solubilized by thermophilic microbes (Sujatha et al. 2004). Citrate is also released P

Table 10.2 Organic acids involved in solubilization of insoluble P synthesized by phosphate solubilizing microorganisms. (Modified from Khan et al. 2010)

Microorganisms	Organic acids produced	References
<i>Burkholderia cepacea</i> DA23 <i>Aspergillus niger</i> <i>Burkholderia</i> , <i>Serratia</i> , <i>Ralstonia</i> and <i>Pantoea</i> <i>Aspergillus flavus</i> , <i>A. candidus</i> , <i>A. niger</i> , <i>A. terreus</i> , <i>A. wentii</i> , <i>Fusarium oxysporum</i> , <i>Penicillium</i> sp., <i>Trichoderma isridae</i> , <i>Trichoderma</i> sp.	Gluconic, citric, oxalic, formic, maleic, gluconic lactic, maleic, malic, acetic, tartaric, citric, fumaric, gluconic	Song et al. (2008); Kumari et al. (2008); Elizabeth et al. (2007); Akintokun et al. (2007)
<i>A. flavus</i> , <i>A. candidus</i>	Glutaric	Akintokun et al. (2007)
<i>Bacillus</i> , <i>Rhodococcus</i> , <i>Arthrobacter</i> , <i>Serratia</i> and one <i>Chryseobacterium</i> , <i>Delftia</i> , <i>Gordonia</i> , <i>Phyllobacterium</i> , <i>Arthrobacter ureafaciens</i> , <i>Phyllobacterium myrsinacearum</i> , <i>Rhodococcus erythropolis</i> and <i>Delftia</i> sp.	Citric acid, gluconic acid, lactic, succinic, propionic	Chen et al. (2006)
<i>Penicillium oxalicum</i>	Malic, gluconic, oxalic	Shin et al. (2006)
<i>Burkholderia glathei</i> (MB 14)	Gluconate and acetate	Kim et al. (2005)
<i>Aspergillus flavus</i> , <i>A. niger</i> , <i>P. canescens</i>	Oxalic, citric, gluconic, succinic	Maliha et al. (2004)
<i>Penicillium rugulosum</i>	Citric, gluconic	Reyes et al. (2001)
<i>Bacillus amyloliquefaciens</i> , <i>B. licheniformis</i> , <i>B. atrophaeus</i> , <i>Penibacillus macerans</i> , <i>Vibrio proteolyticus</i> , <i>xanthobacter agilis</i> , <i>Enterobacter aerogenes</i> , <i>E. taylorae</i> , <i>E. asburiae</i> , <i>Kluyvera cryocrescens</i> , <i>Pseudomonas aerogenes</i> , <i>Chryseomonas luteola</i>	Lactic, itaconic, isovaleric, isobutyric, acetic	Vazquez et al. (2000)
<i>A. niger</i>	Succinic	Vazquez et al. (2000)
<i>Penicillium variabile</i>	Gluconic	Benice et al. (2000)
<i>Pseudomonas cepacia</i>	Gluconic, 2-ketgluconic	Bar-Yosef et al. (1999)
<i>Penicillium rugulosum</i>	Gluconic	Reyes et al. (1999)
<i>Penicillium radicum</i>	Gluconic	Whitelaw et al. (1999)
<i>Penicillium variabile</i>	Gluconic	Vassilev et al. (1996)
<i>A. niger</i>	Citric, oxalic, gluconic	Illmer et al. (1995)
<i>A. awamori</i> , <i>A. foetidus</i> , <i>A. terricola</i> , <i>A. amstelodemi</i> , <i>A. Tamari</i> , <i>Bacillus polymyxa</i> , <i>B. licheniformis</i> , <i>Bacillus</i> sp.	Oxalic, citric	Gupta et al. (1994)
<i>A. japonicus</i> , <i>A. foetidus</i>	Oxalic, citric gluconic succinic, tartaric	Singal et al. (1994)
<i>Penicillium bilaji</i>	Citric, oxalic	Cunningham and Kuyack (1992)
<i>A. niger</i> , <i>P. simplicissimum</i>	Citric	Burgstaller et al. (1992)

from goethite (Geelhoed et al. 1999) or amorphous ferric hydroxides (Dye 1995). Other organic acids such as lactic, isovaleric, isobutyric, acetic, glycolic, oxalic, malonic and succinic acids also generated by the different phosphate solubilizing bacteria (Kpombekou and Tabatabai 1994). Ryan et al. (2001) reported that among the carboxylic acids, dicarboxylic (oxalic, tartaric, malic, fumaric, malonic acids) and tricarboxylic (citric) acids are more effective for P mobilization. According to

Table 10.3 Gram-negative bacteria known to be involved in mineral phosphate solubilization

Gram-negative bacteria	Reference
<i>Acetobacter</i> sp.	Joseph and Jisha (2009)
<i>Acetobacter liquefaciens</i>	Joseph and Jisha (2009)
<i>Acetobacter diazotrophicus</i>	Mahesh Kumar et al. (1999)
<i>Achromobacter xylosoxidans</i>	Jha and Kumar (2009)
<i>Acinetobacter</i> sp.	Rodríguez and Fraga (1999)
<i>Aerobacter aerogenes</i>	Gupta et al. (1998); Rodríguez and Fraga (1999)
<i>Agrobacterium radiobacter</i>	Belimov et al. (1995)
<i>Agrobacterium</i> sp.	Gupta et al. (1998); Rodríguez and Fraga (1999)
<i>Alcaligenes</i> sp.	Gupta et al. (1998); Rodríguez and Fraga (1999)
<i>Arthobacter mysorens</i>	Belimov et al. (1995)
<i>Azotobacter chroococcum</i>	Kumar and Narula (1999)
<i>Brevibacterium</i> sp.	Gupta et al. (1998); Tilak et al. (2005)
<i>Bradyrhizobium japonicum</i>	Antoun et al. (1998)
<i>Burkholderia cepacia</i>	Rodríguez and Fraga (1999)
<i>Burkholderia</i> sp.	Linu et al. (2009)
<i>Cladosporium herbarum</i>	Singh and Kapoor (1999)
<i>Corynebacterium</i> sp.	Gupta et al. (1998); Tilak et al. (2005)
<i>Enterobacter aerogenes</i>	Chung et al. (2005)
<i>Ent. Agglomerans</i>	Kim et al. (1997)
<i>Enterobacter asburiae</i>	Tripura et al. (2007)
<i>Enterobacter cloacae</i>	Goldstein et al. (1999)
<i>Enterobacter intermedium</i>	Kim et al. (2003)
<i>Escherichia freundii</i>	Gupta et al. (1998); Tilak et al. (2005)
<i>E. intermedia</i>	Gupta et al. (1998); Tilak et al. (2005)
<i>Erwinia herbicola</i>	Goldstein and Liu (1987); Goldstein et al. (1993)
<i>Flavobacterium</i> sp.	Goldstein (2001)
<i>Gluconacetobacter</i> sp.	Linu et al. (2009)
<i>Gluconobacter diazotrophicus</i>	Madhaiyan et al. (2004)
<i>Micrococcus</i> sp.	Rodríguez and Fraga (1999) and Gulati et al. (2008)
<i>Mycobacterium</i> sp.	Goldstein (2001)
<i>Pantoea agglomerans</i>	Amellal et al. (1999)
<i>Pantoea dispersa</i> 1A	Selvakumar et al. (2008a)
<i>Pseudomonas pinophilum</i>	Gupta et al. (1998); Tilak et al. (2005)
<i>Pseudomonas corrugate</i>	Pandey et al. (1998)
<i>Pseudomonas aeruginosa</i>	Musarrat et al. (2000)
<i>Pseudomonas aeruginosa</i> GES-18	Tripura et al. (2007)
<i>Pseudomonas cepacia</i>	Babu-Khan et al. (1995)
<i>Pseudomonas fluorescens</i>	Gupta et al. (1998); Tilak et al. (2005)
<i>Pseudomonas putida</i>	Cattelan et al. (1999)
<i>Pseudomonas chlororaphis</i>	Cattelan et al. (1999)
<i>Pseudomonas gladioli</i>	Joseph and Jisha (2009)
<i>Pseudomonas striata</i>	Linu et al. (2009)
<i>Pseudomonas syringae</i>	Tilak et al. (2005)
<i>Pseudomonas trivialis</i> , <i>P. poae</i>	Gulati et al. (2008)
<i>Pseudomonas fragi</i>	Selvakumar et al. (2009b)
<i>Pseudomonas lurida</i>	Selvakumar et al. (2011)
<i>Ralstonia</i> sp.	Perez et al. (2007)
<i>Rahnella aquatilis</i>	Kim et al. (1997)

Table 10.3 (continued)

Gram-negative bacteria	Reference
<i>Rhizobium</i> sp.	Halder and Chakrabarty (1993)
<i>Rhizobium meliloti</i>	Halder and Chakrabarty (1993)
<i>Rhizobium leguminosarum</i> biovar <i>Phaseoli</i>	Chabot et al. (1998)
<i>Rhizobium loti</i>	Halder and Chakrabarty (1993)
<i>Serratia phosphaticum</i>	Gupta et al. (1998); Tilak et al. (2005)
<i>Serratia marcescens</i> GPS-5	Tripura et al. (2007)
<i>Serratia marcescens</i> strain SRM	Selvakumar et al. (2008b)

Illmer and Schinner (1992), gluconic acid may be the most frequent agent of mineral phosphate solubilization.

In general, the ability of different carboxylic anions to desorb P decreases with a decrease in the stability constants of Fe or Al-organic acid complex in the order: citrate > oxalate > malonate/malate > tartrate > lactate > gluconate > acetate > formate (Ryan et al. 2001). This result serves to confirm the ability of the strains in mobilizing P from insoluble sources, in particular those producing altogether citrate, malate and tartrate. The solubilization of 837 mg l⁻¹ CaHPO₄ by *P. bilaiae* was achieved at pH 4.5 in the presence of citrate, but no CaHPO₄ solubilization occurred at the same pH in the presence of the inorganic acid alone indicating that chelation involved citric acid (Cunningham and Kuiack 1992). Gluconic acid or *P. radicum* inoculation alone solubilized more amorphous Al-P than HCl at the same pH (Whitelaw et al. 1999).

The insoluble sources of inorganic P in liquid broth solubilized by PSM accompanied by the production of organic acids: the action of organic acids synthesis and lowering the pH cause dissolution of P compounds (Banik and Dey 1982; Kucey 1988; Cunningham and Kuiack 1992; Whitelaw 2000; Pradhan and Sukla 2005). The production of organic acid leads to acidification of microbial cells and their surroundings and consequently, the release of P ions from the P mineral by H⁺ substitution for Ca²⁺ (Goldstein 1994). Organic acids produced by PSM determined by methods such as high performance liquid chromatography (HPLC) and enzymatic methods (Parks et al. 1990; Whitelaw 2000). Earlier studies on this phenomenon were restricted to mesophilic temperatures (Chung et al. 2005; Chen et al. 2006). Gluconic acid, the principal organic acid produces by *Pseudomonas* sp. (Illmer and Schinner 1992), *Erwinia herbicola* (Liu et al. 1992), *Pseudomonas cepacia* (Goldstein et al. 1993) and *Burkholderia cepacia* (Rodríguez et al. 2000a) *Rhizobium leguminosarum* (Halder et al. 1990), *Rhizobium meliloti* (Halder and Chakrabarty 1993) and *Bacillus firmus* (Banik and Dey 1982) produce noticeable amounts of 2-keto gluconic acid.

10.5.3 Genes Responsible for Bacterial P Solubilization

Bacterial mineral phosphate solubilization mainly attributed to the activity of glucose dehydrogenase; a membrane bound enzyme that is involved in the direct

oxidation of glucose to gluconic acid (Goldstein 1995; Sashidhar and Podile 2010). Subsequently gluconic acid is enzymatically converted to 2-keto gluconic acid and 2,5-diketogluconic acid. The 2-keto gluconic acid is more effective than gluconic acid in solubilizing phosphate (Kim et al. 2002).

The conversion of glucose to gluconic acid by glucose dehydrogenase (GDH) is involved in this process, as is its oxidation to 2-keto gluconic acid via the activity of gluconate dehydrogenase (Goldstein 1994). Glucose dehydrogenase requires pyrroloquinoline quinone (PQQ) as a redox cofactor. Genes encoding for the enzymes inherent to PQQ biosynthesis cloned from *Klebsiella pneumoniae* and consisted of six open reading frames (*pqqA* to *pqqF*) (Meulenberg et al. 1992). This assumption been corroborated by cloning of two genes involved in gluconic acid production, PQQ synthase (Goldstein and Liu 1987; Liu et al. 1992; Rodríguez et al. 2000b) and *gabY* (Babu-Khan et al. 1995) genes. Genetic basis of mineral phosphate solubilization not well understood. Because the production of organic acids considered as the principal mechanism for mineral phosphate solubilization, it could assumed that any gene involved in organic acid synthesis might have an effect on this character. Goldstein and Liu (1987) cloned a gene from *Erwinia herbicola* that is involved in mineral phosphate solubilization by screening the antibiotic-resistant recombinants from a genomic library in a medium containing hydroxyapatite as the source of P. The expression of this gene allowed production of gluconic acid and mineral phosphate solubilization activity in *E. coli* HB101. Sequence analysis of this gene (Liu et al. 1992) suggested its probable involvement in the synthesis of the enzyme pyrroloquinoline quinone (PQQ) synthase, which directs the synthesis of PQQ, a co-factor necessary for the formation of the holoenzyme glucose dehydrogenase (GDH)-PQQ. This enzyme catalyzes the formation of gluconic acid from glucose by the direct oxidation pathway. Following a similar strategy, a mineral phosphate solubilization gene from *Pseudomonas cepacia* was isolated (Babu-Khan et al. 1995). The expression of gene (*gabY*) also allowed the induction of the mineral phosphate solubilization phenotype via gluconic acid production in *Escherichia coli* JM109, showed no apparent homology with the previous cloned PQQ synthetase gene (Liu et al. 1992; Goosen et al. 1989), but it did with a permease membrane protein system. The *gabY* gene could play an alternative role in the expression and/or regulation of the direct oxidation pathway in *Pseudomonas cepacia*, thus acting as a functional mineral phosphate solubilization gene in-vivo.

In fact, the information about the genetic or biochemical mechanisms involved in the synthesis of the GDH-PQQ holoenzyme is scant, and variations between constitutive and inducible phenotypes observed among several bacterial species (Goldstein 1994). A *pqq* gene cluster producing *Pqq* detected in *E. intermedium* and this sequence conferred phosphate-solubilizing activity to *E.coli* DH5 α . The 6783 bp *pqq* sequence had six open reading frames (*pqq* A, B, C, D, E and F) showed 50–95 % homology to *pqq* gene of other bacteria. Kim et al. (2003). Studies shows that 2-keto gluconic acid (pKa=2.66) is more effective then gluconic acid (pKa=3.41) in solubilizing phosphate (Kim et al. 2002). Glucose, gluconate, mannitol, and glycerol are among the possible inducers of the holoenzyme activity (Van Schie et al. 1987).

10.6 Role of P Solubilizing and Mobilizing Microorganisms in Crop Production

Phosphate rock minerals are often too insoluble to provide sufficient P for crop uptake. Experiment conducted by Yazdani et al. (2009) on plant growth promoting rhizobacteria (PGPR) on yield and yield components of corn (*Zea mays* L.), showed that farmyard manure application increased row number, ear weight, grain number per year, grain yield, biological yield and harvest index compared to check. Furthermore, using of PSM and PGPR in addition to conventional fertilizer applications (NPK) could improve ear weight, row number and grain number per row and ultimately increased grain yield in green manure and check plots. According to results in all fertilizer treatments application of PSM and PGPR together could reduce P application by 50 % without any significant reduction of grain yield. However, this treatment could not compensate 50 % reduction of N application. Use of PSMs can increase crop yields up to 70 % (Verma 1993). Combined inoculation of arbuscular mycorrhiza and PSB give better uptake of both native P from the soil and P coming from the phosphatic rock (Goenadi et al. 2000; Cabello et al. 2005). The varying success of PSM inoculations depends on insufficient survival and colonization of inoculated strains, competition with native microorganisms, nature and properties of soils and plant varieties, starvation of nutrients in the rhizosphere to produce enough organic acids to solubilize oil phosphates and inability of PSMs to solubilize soil phosphates (Kucey et al. 1989; Gyaneshwar et al. 2002).

Afzal et al. (2005) studied the effect of phosphate solubilizing microorganisms on phosphorus uptake, yield and yield traits of wheat (*Triticum aestivum* L.) in rainfed area and reported significant increased in grain yield and biological yield by the treatments and maximum yield was recorded when PSM was used with phosphorus alone or along with organic matter. PSM alone or along with other combinations produced profound effect on grain and biological yield, tillers per squaremeter and seed phosphorus content. *Pseudomonas* spp. enhanced the number of nodules, dry weight of nodules, yield components, grain yield, nutrient availability and uptake in soybean crop (Son et al. 2006). Phosphate solubilizing bacteria enhanced the seedling length of *Cicer arietinum* (Sharma et al. 2007) while co-inoculation of PSM and PGPR reduced P application by 50 % without affecting corn yield (Yazdani et al. 2009). Inoculation with PSB increased sugarcane yield by 12.6 % (Sundara et al. 2002). Sole application of bacteria increased the biological yield while the application of the same bacteria along with mycorrhiza achieved the maximum grain weight (Mehrvaz et al. 2008). Single and dual inoculation along with P fertilizer was 30–40 % better than P fertilizer alone for improving grain yield of wheat, and dual inoculation without P fertilizer improved grain yield up to 20 % against sole P fertilization (Afzal and Bano 2008). Mycorrhiza along with *Pseudomonas putida* increased leaf chlorophyll content in barley (Mehrvaz et al. 2008). Rhizospheric microorganisms can interact positively in promoting plant growth, as well as N and P uptake.

Seed yield of green gram enhanced by 24 % following triple inoculation of *Bradyrhizobium* + *Glomus fasciculatum* + *Bacillus subtilis* (Zaidi and Khan 2006).

Growth and phosphorus content in two alpine *Carex* species increased by inoculation with *Pseudomonas fortinii* (Bartholdy et al. 2001).

The beneficial effects of inoculation of seed or soil with phosphate solubilizing bacteria resulting in higher crop yields in various crops due to improved solubilization of fixed phosphorus and applied phosphates is well known (Yadav and Dadarwal 1997; Viveganandhan and Jahuri 2002) (Table 10.4).

Srivastav et al. (2004) have proposed the utility of P solubilizing *Pseudomonas* sp. as biocontrol agents for the control of soil borne plant pathogens. An increase in P availability to plants through the inoculation of phosphate solubilizing microorganisms has reviewed under pot and field conditions (Chabot et al. 1996; Kumar et al. 2001). There are several studies indicating that soil inoculation with phosphate solubilizing bacteria has shown to improve solubilization of fixed soil P and applied phosphates resulting in higher crop yields (Nautiyal et al. 2000; Hameeda et al. 2008). A yield increase is not always combined with higher P uptake (Reyes et al. 2002). An alternative approach for the use of phosphate-solubilizing bacteria as microbial inoculants is the use of mixed cultures or co-inoculation with other microorganisms. In this regard, some results suggest a synergistic interaction between vesicular arbuscular mycorrhiza (VAM) and PSB, which allows for better utilization of poorly soluble P sources (Ray et al. 1981; Piccini and Azcón 1987; Garbaye 1994; Toro et al. 1996; Frey-Klett et al. 1997; Toro et al. 1997; Kim et al. 1998). Similarly, plant growth can be increased by dual inoculation with PSB and *Azospirillum* (Alagawadi and Gaur 1992; Belimov et al. 1995) or *Azotobacter* (Kundu and Gaur 1984; Monib et al. 1984).

10.7 Bioinoculant Production

“Bacterial inoculant” is a formulation containing one or more beneficial bacterial strains (or species) in an easy-to-use and economical carrier material organic, inorganic or synthesized from defined molecules. The desired effects of the inoculant on plant growth can include nitrogen fixation in legumes, biocontrol of (mainly) soil-borne diseases, the enhancement of mineral uptake, weathering of soil minerals and nutritional or hormonal effects. “Biofertilizer” is a misleading but widely used term meaning “bacterial inoculant”. Usually it refers to preparations of microorganism(s) that may be a partial or complete substitute for chemical fertilization (like rhizobial inoculants). The reason for using the word “fertilizer” is that in some countries it allows easier registration for commercial use.

Microorganisms with potential as plant growth promoters used to produce inoculants. A great interest in the use of microorganisms as bioinoculants exists especially in areas with a low P availability results an unfavorable soil pH. In addition, inoculants are used to improve the fertilizer efficiency of rock phosphate (Goenadi et al. 2000; Reddy et al. 2002). Because of the ubiquitous occurrence of phosphorus-mobilizing microbes, a yield increase by inoculation with additional strains may be beneficial if these organisms possess different growth-promoting

Table 10.4 Inoculation effect of mineral phosphate solubilizing bacteria on nutrient uptake and yield of different crops. (Modified from Sindhu et al. 2009 In: Phosphate solubilizing microbes for crop improvement © Nova Science Publishers, Inc. New York)

Conditions	Crop	Bacteria	Response	Reference
Greenhouse	Bamboo	<i>Enterobacter cloacae</i> , <i>Burkholderia cepacia</i> and <i>Serratia marcescens</i>	Increased dry matter	Maheshkumar (1997)
	Canola	<i>Pseudomonas putida</i>	Increased P uptake and yield	Lifschitz et al. (1987)
	Rice	<i>Pseudomonas striata</i>	Increased P uptake and yield	Monod et al. (1989)
	Wheat	<i>Pseudomonas striata</i> and <i>Bacillus polymyxa</i>	Increased P uptake and yield	Kundu and Gaur (1980)
	Wheat	<i>Azospirillum lipoferum</i> and <i>Bacillus megaterium</i>	Increased shoot P and shoot weight	El Komy (2005)
	Maize	<i>Pseudomonas fluorescens</i>	Increased grain yield and P content	Henri et al. (2008)
	Chickpea	<i>Pseudomonas</i> sp.	Increased P uptake and dry matter	Krishnaraj (1996)
Field	Mungbean	<i>Bacillus subtilis</i> , <i>B. circulans</i> and <i>Aspergillus niger</i>	Increased nodulation and grain yield	Gaund and Gaur (1991)
	Groundnut	<i>Pseudomonas striata</i>	High pod yield and P uptake	Agasimani et al. (1994)
	Soybean	<i>Pseudomonas striata</i>	Increased yield and P content	Dubey (1997)

abilities, for instance N₂ fixation, phyto hormone production and phosphorus mobilization (Peix et al. 2001). Phosphorus bio inoculants can assist in increasing the availability of accumulated phosphates for plants by solubilization (Khan et al. 2007).

Potential materials that are able to support good growth and survival of bacteria are need in inoculants production. Many materials have been evaluated, including different types of coals, bentonite, corn oil, sawdust (Kostov and Lynch 1998), mineral soils, peat, peat moss, vermiculite, perlite and alginate (Trivedi et al. 2005). Peat is commonly used material for inoculant carrier. Finely ground peat is most commonly use in conventional inoculant production. There have been reports on the difficulty of obtaining autoclaved or gamma-irradiated peat as carriers (Machi 2006). The high temperature during steam sterilization or the high dosage needed for irradiation might generate toxic substances for bacteria. However, perlite can be easily sterilize with no risk of producing toxic substances because it is a volcanic stone composed of a little-hydrated aluminium silicate. The agronomic use of agro-wastes as substrates causes changes in the soil affecting its physico-chemical characteristics and microbial activity in the rhizosphere. The breakdown of such materials to simple sugars provides energy sources for heterotrophic microorganisms such as P-solubilizing and nitrogen fixing bacteria. Normally, the growth and

metabolic activity of soil microorganisms are limited by the availability of nutrients. Therefore, several kinds of agrowastes such as rice straw compost will be good carrier materials for the inoculants and improver of soil condition. However, the peat moss with phosphate solubilizers showed good survival of inoculants and effects on crops. However, their performance is severely affected by environmental conditions especially under stress conditions and influencing plant growth promotion (Tilak 1991). Though a number of PGPB have been reported (Podile and Kishore 2006), very little is known about cold tolerant plant growth promoting bacteria and their performance under cold growing conditions. Moreover among various soil productivity constrain, phosphorus availability plays a very crucial role in crop production. Water soluble phosphorous applied in such acid soils is rapidly fixed to unavailable forms and accounts for the low phosphate use efficiency (Pal 1998). While some progress has been made in this direction, mainly from the N.W. Indian Himalayan Region (Pandey et al. 2006), the vast majority of alpine and sub-alpine regions have largely remained unexplored.

A great interest in the use of microorganisms as biofertilizers exists especially in areas with a low P availability because of an unfavorable soil pH. In addition, inoculates are used to improve the fertilizer efficiency of rock phosphate (Goenadi et al. 2000; Reddy et al. 2002). Although mycorrhizal fungi are able to improve the phosphorus supply of higher plants more than other microbes, their use as biofertilizers is complicated because AM fungi, which are able to infect many arable crops, are obligate symbionts. Up-to-date methods for an in-vitro production of inoculant do not exist. In addition, P fertilization depresses the formation of arbuscular mycorrhizas. Therefore, the use of AM inoculate is mostly confined to horticulture and recultivation of mine areas. Much research focuses on bacteria and fungi, which live in association with higher plants. Some bacterial inoculants can enhance root colonization by mycorrhizal fungi (Ratti et al. 2001). Although many authors report a growth-promoting effect of phosphorus solubilizing microorganisms (PSM) (Narula et al. 2000; Sundara et al. 2002), results in the field are highly variable (Gyaneshwar et al. 2002). A yield increase is not always combined with higher P uptake (Deubel et al. 2002; Reyes et al. 2002). The varying success of PSM inoculations can be due to different reasons (Kucey et al. 1989; Gyaneshwar et al. 2002) survival and colonization of strains, competition with native microorganisms, soils properties and plant varieties, nutrients in the rhizosphere and production of organic acids. Because of the ubiquitous occurrence of phosphorus-mobilizing microbes, a yield increase by inoculation with additional strains may be beneficial if these organisms possess different growth-promoting abilities, for instance N_2 fixation, phyto hormone production and phosphorus mobilization (Peix et al. 2001). One of the greatest problems is insufficient selection and test methods for phosphorus-mobilizing microorganisms. The selection on clear zones or a pH decrease in simple plate tests cannot reflect the real P binding capabilities under soil and rhizosphere conditions. A better possibility maybe the selection of microbes, which can effectively use P adsorbed on goethite or other minerals (He et al. 2002). Because many more microbes have the ability to solubilize phosphates under special conditions than to colonize and promote growth of higher plants, it is useful to select first

based on growth-promoting abilities. A limited number of strains can then be tested for special properties. It must be taken into account that test conditions influence the results to a large extent. A given strain can respire a sugar to CO₂ under high oxygen supply or produce carboxylic acids by fermentation or incomplete respiration if oxygen is limiting. Moreover, the type of C sources affects the production of microbial metabolites (Kim et al. 1998; Deubel et al. 2000). Our earlier explorations in this region, revealed the presence and utility of novel cold tolerant plant growth promoting bacterial species viz., *Pantoea dispersa*, *Serratia marcescens*, and *Exiguobacterium acetylicum* (Selvakumar et al. 2008a, b, 2009c). Fluorescent Pseudomonads are predominant colonizers of the plant rhizosphere and play a definite role in nutrient mobilization and disease control. Though the diversity of Pseudomonads in the rhizospheric soils of the Indian Himalayan Region (IHR) has been documented (Gulati et al. 2008; Mishra et al. 2008, 2009).

10.8 Future Prospects

The cold environments are extremely diverse in nature with fluctuation in temperature, radiation and pressure. These environments present largely “untapped” biore-source and the microorganisms flourishing, there have great potential for isolation of new and novel microorganisms. Exploitation of cold tolerant PSB-plant interactions can result in the promotion of plant health and can play a significant role in low-input sustainable agriculture applications for both food and non-food crops. The availability of complete genome sequences of key cold tolerant P solubilizing bacteria would be helpful in the identification of genes governing P solubilization and colonization and establishment of PSB bacteria in the plant rhizosphere. This information will form the foundation for transcriptome and proteome analysis currently optimized in studying other plant–microbe interactions. An understanding of the mechanisms of P solubilization enabling these cold tolerant PSB bacteria to interact with plants will be essential to fully achieve the biotechnological potential of efficient plant-bacterial partnerships for a range of applications. One promising area of research for future studies is developing cold tolerant P solubilizing bacterial consortium (and rhizobacteria) to promote the sustainable production of crops.

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Chapter 11

Osmotolerant Microbial Resources of Saline Ecologies of India: Dynamics and Potential

Jayashree Rath and T. K. Dangar

Abstract In India, about 1.7 m ha saline, 3.8 m ha sodic-saline and 1.2 m ha coastal saline area and, about 6749 km² mangroves spread on coastal and Island regions constitute the saline ecologies. The mangroves are fragile but highly important ecological entities, which have great biodiversity and novel natural resources including the microbes. The coastal and inland salinity severely affect productivity. However, every ecological niche has well-adapted resident microbial guilds which maintain the ecosystem functioning. Researches on diverse saline ecologies of India have led to identify osmotolerant beneficial microbes, which can be exploited to maintain environmental health, sustain agricultural, and commercial production of novel biological products, food and other industries, which operate in hypersaline condition. The osmotolerant microbes endure osmotic shock by moderating Na⁺/K⁺ flow, accumulating osmolytes or regulating the functions of stress tolerant enzymes (osmozymes). Unlike stress-free mesophilic counterparts, the osmotolerant microbes are more versatile as they can function in both stress and stress-free conditions. Therefore, the microbial resources of the saline ecologies of the country have the potency for welfare of the biological and environmental health, and productivity.

11.1 Introduction

Soil is a complex, heterogeneous and discontinuous microhabitat that harbours five major groups of microorganisms e.g., bacteria (>6000 genomes/g soil), actinomycetes, fungi, algae and protozoa which count about 10⁹, 10⁸, 10⁶, 10⁵ and 10⁴ cultivable organisms i.e., colony forming units (cfu)/g soil, respectively, and mediate about 80–90 % of soil functionalities (Liesack et al. 2000; Watve et al. 2000; Nanipieri et al. 2003; Sharma et al. 2005; Subba Rao 2007; Alexander 2011). Besides the high and low temperature regions; the salinity along the eastern and western

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coastlines of the country, inland saline regions, sodic and acidic saline soils, salt lakes, the mangroves that form the vast wet and saline habitats along different intertidal zones of India and other continents too (excepting Europe, the Arctic and Antarctic regions) is one of the major abiotic stresses that limit crop growth, decrease plant dry matter accumulation, leaf area, metabolic pathways like photosynthesis, respiration, nitrogen fixation, carbohydrate metabolism etc. (Yadav and Yadav 2003; Chen et al. 2008; Amirjani 2011) which hamper both agricultural and non-agricultural productivity. Microbes are the geoengineers/biogeochemists which keep the bio-geochemical processes, such as C, N, S, P, K, Fe, Zn, Mn etc. cycles operational in the soil, metabolize plant growth regulators (IAA, GA, cytokinin, ABA and phenolics), toxins, inhibitors etc., exert plant growth promotion (PGP) functions and sustain plant growth and development (Bhattacharjee et al. 2008; Sahoo et al. 2013a, b, c).

The osmotolerant microbes possessing PGP properties would maintain the health and functionalities to sustain productivity of the native ecology and crops of the saline regions. Therefore, comprehension of the microbial structural, functional and genetic diversity, interactions among themselves and with antimicrobial chemicals/pesticides, stress (osmotic) tolerance, toxin/inhibitor production by antagonistic/biocontrol microbes of the pests/diseases etc. of the coastal/inland/mangrove saline ecologies would be most effective proposition to exploit the polyvalent osmotolerant microbes possessing PGP and biocontrol functions to maintain soil health, and suppression of the pests and diseases of the normal and stressed soil ecologies to overcome the production constraints.

11.2 Area and Distribution of Saline, Sodic-Saline and Alkaline Soils in India

Salinity of any system varies with space and time (higher in summer) but it is a critical constraint in the arid, semi-arid, estuarine, coastal and mangrove, inland saline, salt marsh, salt lakes and salt pans (Gauda and Panigrahy 1989; CSSRI 2012; Singh et al. 2012). Salinity causes major agricultural problem in many parts of the world, especially for irrigated crops (Gamalero et al. 2009; Zarea et al. 2012). Various authors have differently classified the saline soils for various purposes and the estimates of saline areas vary widely with the estimation methods (Dagar 2005; Dagar and Singh 2007; Maji et al. 2010). Maji et al. (2010) grouped the saline soils into different degraded categories viz., exclusive saline, eroded saline, acid saline, saline soils under wind erosion, saline soils under open forest and waterlogged saline soils which cover 2.6, 0.04, 0.02, 0.1, 0.06 and 0.03 million hectare (m ha) area, respectively. Depending on physico-chemical properties and nature of the salts, the saline soils are classified into 27 sub-groups but for practical purposes, they are grouped into five categories viz., saline, alkaline, sodic, saline-alkaline and saline-sodic types which are grossly spread over 9.38 m ha area comprising of 5.5 m ha saline (including coastal saline) and 3.88 m ha alkaline soil regions (Dagar 2005). However, Dagar and Singh (2007) reported that salinity and alkalinity have

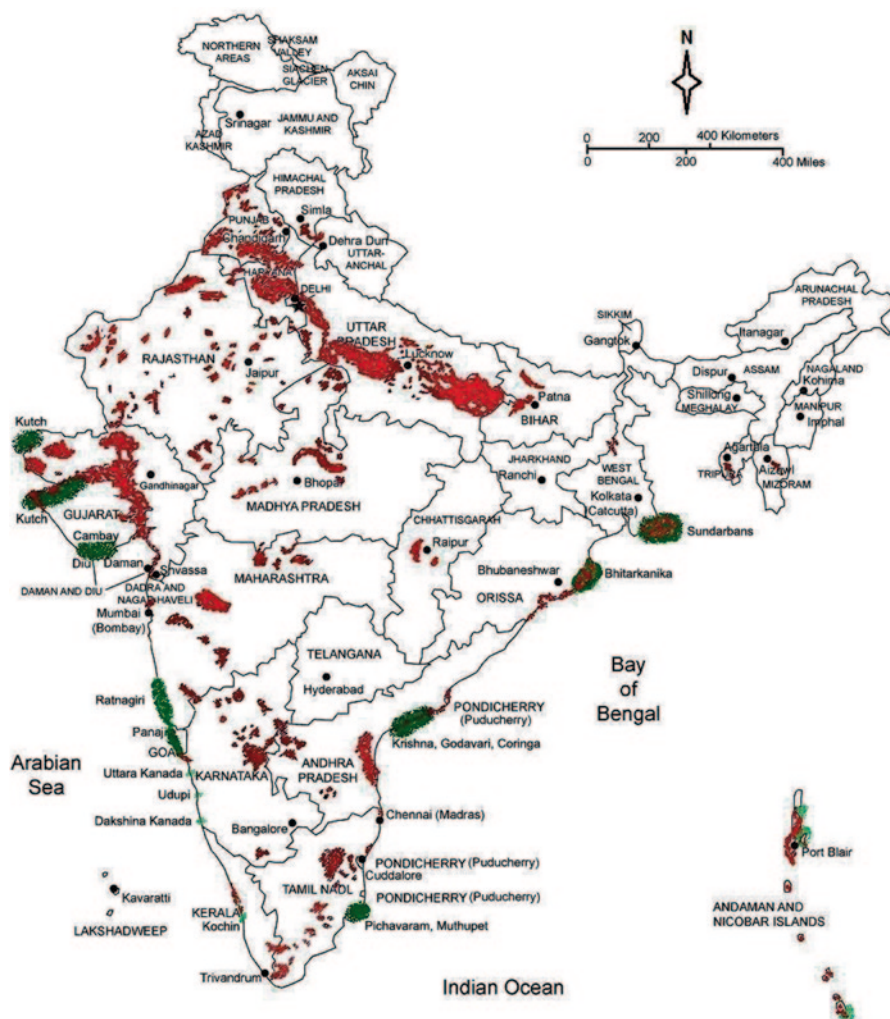


Fig. 11.1 Diagrammatic view of distribution of mangroves (green color) and saline areas (red color) in India

degraded about 8.55 m ha area. Maji et al. (2010) mapped the saline soils of India and recorded 3.0 m ha saline and 3.8 m ha sodic soils i.e., 6.8 m ha total salt affected regions. More recent report showed that salinity affected soils in India cover 1.7 m ha saline, 3.8 m ha sodic-saline and 1.2 m ha coastal saline i.e., altogether salinity has occupied 6.7 m ha area (CSSRI 2012). Furthermore, India has about 1.2 m ha (5700 sq. km) coastal saline zone (Gauda and Panigrahy 1989; Dagar 2005; CSSRI 2012) and 6749 km² mangroves spread along the coasts of mainland and the Islands (Mishra et al. 2012a, b; Singh et al. 2012). The coastal/Island saline and mangrove regions are important ecological entities, as they have great biodiversity and novel natural resources (Fig. 11.1, Table 11.1).

Table 11.1 Extent and distribution of major salt affected soils in India. (Adapted from CSSRI, Kernal, India (2012))

State	Saline soils (ha)	Sodic soils (ha)	Coastal saline soil (ha)	Total (ha)
Andhra Pradesh	0	196,609	77,598	274,207
Andaman and Nicobar Islands	0	0	77,000	77,000
Bihar	47,301	105,852	0	153,153
Gujarat	1,218,255	541,430	462,315	2,222,000
Haryana	49,157	183,399	0	232,556
Jammu and Kashmir	0	17,500	0	17,500
Karnataka	1307	148,136	586	150,029
Kerala	0	0	20,000	20,000
Maharashtra	177,093	422,670	6996	606,759
Madhya Pradesh	0	139,720	0	139,720
Orissa	0	0	147,138	147,138
Punjab	0	151,717	0	151,717
Rajasthan	195,571	179,371	0	374,942
Tamil Nadu	0	354,784	13,231	368,015
Uttar Pradesh	21,989	1,346,971	0	1,368,960
West Bengal	0	0	441,272	441,272
Total	1,710,673	3,788,159	1,246,136	6,744,968

11.3 Physico-Chemical Perception of Saline Soils in India

Salt stress differently affects plants but extreme stress is fatal and moderate to low stress affect growth, morphology, physiology or biochemistry of the plants. Soil salinity beyond electrical conductivity (EC) ~ 4 dS/m is adjudged moderate but > 8 dS/m is high salinity stress while pH 8.8–9.2 is non-stress, 6–8.7 and 9.3–9.7 are moderate and $\leq 4.2 \geq 9.8$ are high stress for rice (Zahran 1997; Gamalero et al. 2009). Salt-tolerant species can tolerate 7–8 dS/m E_{Ce}/E_{Ciw} (soil/irrigation water) (Aronson 1989) and a wide range of species which grow well in natural saline habitats with E_{Ce} 8 or > 8 dS/m are designated as halophytes. For management purposes, this criterion appears to be relevant (Dagar and Singh 2007).

Salinity is the result of all non-carbonate salts dissolved in water. The salinity level in seawater is about 35 ppt (35,000 mg/L or 3.5%), while brackish estuaries may have salinity levels between 1 and 10 ppt. Salinity is determined from chloride concentration i.e., Salinity (ppt) = $0.0018066 \times \text{Cl}^-$ (mg/L). Electrical conductivity of soil extract exceeding 4000 ($\mu\text{S}/\text{cm}$), exchangeable sodium less than 15% and the sodium adsorption ratio of < 13 designate a soil as saline soil. Soil salanization occurs either by natural processes (primary salanization) or human-induced processes (secondary salanization). Primary salanization is the result of weathering of native rocks, fossil or entrapped solutions of earlier marine sediments transported away through groundwater streams (Spark 1995). Deforestation, construction of reservoirs, salt farming, over use of ground water, irrigation using saline water, erosion and engineering works etc. (Mitsuchi et al. 1986) cause secondary salanization. The soil structure and salinity are the main agents, controlling distribution of the

mangroves. The salinity of the soil depends upon tidal flow, rainfall, texture of soil, presence of vegetation, elevation area, proximity to freshwater inflow etc.

Saline soils are mostly sodic due to predominance of sodium chloride but do not attain higher alkalinity. Usually, the natural saline environments (4–30% NaCl level) are widely distributed in arid and semiarid regions, contain Na^+ (predominant), K^+ , Mg^{2+} and Ca^{2+} cations; higher free chlorides (mainly), few sulfates, nitrates, carbonates and bicarbonate anions and electrical conductivity (EC) >4 dS/m, exchangeable sodium (%) (ES) >15 and pH 8.5–10.7, but poor in soil structure, nitrogen, phosphorus and zinc contents (Zahran 1997; Zahran et al. 1992). The pH of hypersaline ecosystems is usually <8.5 as the soluble salts are mainly neutral and exchangeable sodium is $<15\%$, presence of variable amounts of ammonia and nitrate, and the divalent cations like Mg^{2+} and Ca^{2+} concentrations are either low or absent (Zahran 1997; Zahran et al. 1992). In limited organic matter containing hypersaline soil, sulfate acts as electron acceptor during anaerobic mineralization which prevails in the organic poor Indian saline soils.

In acidic soils, ESP is <15 and pH is much lower than in sodic soils. Based on the range of pH values, the categories of acid soil groups are: strongly acidic (pH <4.5); moderately acidic (pH 4.5–5.5); slightly acidic (pH 5.5–6.5) and non-acidic (pH >6.5).

Owing to excess salts ($\text{ECe} > 4$ dS/m) and absence of significant amount of sodium ($\text{ESP} < 15$), saline soils are generally flocculated and their conductivity is equal to or even greater than their non-saline counterparts. A saline-alkali soil ($\text{ECe} > 4$ dS/m; $\text{ESP} > 15$) was described similar to that of saline soils as long as sufficient salts are present, whereas, upon leaching, these soils become alkaline (pH > 8.5) leading to dispersion and their permeability reduces to the level that affects crop growth (Dagar and Singh 2007).

11.4 Diverse Saline Ecologies in India

India is a vast country bordered with two seas on the eastern and western coasts and the Himalayas on the North. The geography and topology of the country have created wide ecological variation including the saline habitats. The types and features of the saline ecologies of the country are briefly but comprehensively outlined below.

11.4.1 Coastal Saline Plain

India has about 1.2 m ha (5700 sq. km) coastal saline zone spreading over 7517 km i.e., about 2700 km in the East coast along the Bay of Bengal and 3000 km in the West coast along the Arabian Sea and 1816.6 km Island territories (Fig. 11.1, Table 11.1) (Gauda and Panigrahy 1989; Dagar 2005; CSSRI 2012). The two coast lines are spread about 150 m mean sea level (MSL) over a narrow i.e., 10 to 25 km

belt of the Arabian Sea coast in the West extending from Surat to Kanyakumari covering Kerala, Karnataka, Konkan, Kachchh (Kutch or Kachh), Gujarat and Kathiawar peninsula, and the Bay of Bengal coast in the East covering West Bengal, Orissa, Andhra Pradesh (including a broad Godavari and Krishna delta) and Tamil Nadu coast (Yadav and Yadav 2003; Tripathi et al. 2007; Maji et al. 2010).

11.4.2 The Mangrove Ecologies

Mangroves (or mangals) are the salt tolerant forest ecosystems distributed in 112 countries and territories, largely confined to 30° North and South of the equator (Bandaranayake 2002), occupy 181,000 km² area i.e., over a quarter of world coastline and three quarters of tropical coastlines which are fragile ecological entities but have high productivity, great biodiversity and novel natural resources (Fig. 11.1, Table 11.1) (Kumar 2000; Dagar and Singh 2007; Sahoo and Dhal 2009; Mishra et al. 2009, 2011, 2012a, b; Singh et al. 2012). In India, mangroves occupy 0.1 % of the total geographical area of the country and 5 % of the world's mangrove vegetation (Dagar and Singh 2007; Mishra et al. 2012a, b; Singh et al. 2012). India has about 6749 km² mangrove forests spread along both the coasts of the main land (4662.56 km²) and the Islands (about 2087 km²) (Mishra et al. 2012a, b; Singh et al. 2012). Out of about 6749 km² mangroves in India, 80 % are present along the East coast (West Bengal to Tamil Nadu), i.e., the Sundarbans (largest in size and biodiversity), Bhitarkanika, Krishna-Godavari delta, Pichavaram and the Andaman and Nicobar mangroves, and the remainder 20 % mangroves are scattered along the West coast from Kutch to Kerala. Mangrove cover area (km²) of different states/territories is highly variable viz., Andhra Pradesh 329, Goa 15, Gujarat 936, Karnataka 3, Kerala 8, Maharashtra 158, Orissa 203, Tamil Nadu 35, West Bengal 2118, Andaman and Nicobar Islands 637, Daman and Diu 617, Puducherry 1 and Minicoy has 500 m² mangrove area (Table 11.2, Fig. 11.1) (Kumar 2000; Dagar and Singh 2007; Singh et al. 2012).

11.4.2.1 The Sundarbans Mangrove Ecology

The Sundarbans (21°30'–22°40'N, 88°05'–89°55'E, MSE 0–10 m) spread over South 24-Parganas in West Bengal, India and Khulna division in Bangladesh are world's largest coastal wetland mangrove forest covering about one m ha area of numerous islands formed by the sediments deposited in the delta regions of three major rivers viz., The Ganga, Brahmaputra and Meghna, and a minor one viz., Harinbhanga (Ichamati or Raimongal in Bangladesh) which demarcates the area between India (9630 km², 599,330 ha) and Bangladesh (426,300 ha) (Gopal and Chauhan 2006) (Fig. 11.1, Table 11.2).

The soils of the Sundarbans mangroves contain 0.02–0.09 % nitrogen, 0.06–0.1 % phosphate, 0.1–0.5 % potash and 0.5–1.05 % of total carbon. During summer

Table 11.2 Area and distribution of mangroves in India

State/Union territory	Name of mangrove	1987 (000 ha)	1997 (000 ha)	2012 (000 ha)
West Bengal	Sundarbans	420	212.3	215.5
Orissa	Bhitarkanika	15	21.1	22.2
Andhra Pradesh	Godavari, Krishna, Coringa	20	38.3	35.2
Tamil Nadu	Pichavaram, Muthupet	15	2.1	3.9
Kerala	Kochin	Sparse	Nil	0.6
Karnataka	Kundapura, Nushikote	6	0.3	0.3
Goa	Chorao	20	0.5	2.2
Maharashtra	Ratnagiri	33	12.4	18.6
Gujarat	Gulf of Combay, Gulf of Kutch	26	99.1	105.8
Andaman and Nicobar Islands	Andaman and Nicobar Islands	119	96.6	61.7
Daman and Diu	Nil	NA	NA	61.7
Puducherry	Puducherry	NA	NA	0.1
	Total	674	482.7	527.8

NA not available

seasons, salinity increases in the surface soil due to evaporation of soil moisture, whereas, it is low in the monsoons. However, Hossain et al. (2012) reported that the Sundarbans soil is clayey loam to silty-sand, moisture 9.0–27.0%, pH 7–8, salinity 0.293–4.58 mS/cm, total nitrogen 0.057–0.158%, available nitrogen 0.504–2.016 µg/g soil, soil salinity 20.99–34.99 mg chloride/g soil and organic carbon 0.460–0.885%.

11.4.2.2 The Bhitarkanika and Mahanadi Delta Mangrove Ecologies

The Bhitarkanika (20°30'–20°50'N and 86°30'–87°6'E) mangroves extend about 139.39 km² in the Brahamani and Baitarani deltaic regions in Orissa (Fig. 11.1, Table 11.2) (Mishra et al. 2012a, b). Mishra et al. (2012a, b) recorded pH 6.02 (winter)—7.89, redox potential (EC) 6.4–19.5 mS/cm, total N 200.6–285.5 kg/ha, P 9.0–24.0 kg/ha, K 1053–2378 kg/ha, total C 0.11–0.59% and the soil is grain silt or clayey at five different sites. However, at four other locations of Bhitarkanika, ranges of the physico-chemical conditions viz., pH 6.95–7.36, EC (mS/cm) 10.85–12.5, available N (kg/ha) 224.17–260.05, available P (kg/ha) 11.5–14.21, available K (kg/ha) 1062.0–2224.0 and total C (%) 0.25–0.40 were reported by Thatoi et al. (2012).

The Mahanadi delta mangrove area (20°15'–20°70'N and 87°–87°40'E) is bounded by the rivers Mahanadi, Hansua (a Brahamani tributary) and Jumboo (Behera et al. 2012). The soil has pH 5.52–6.70, salinity 0.5–1.04% NaCl, organic carbon 0.65–0.80%, as well as, N, P and K (kg/ha) 215–285, 9.32–23.0 and 470.42–496.00, respectively (Behera et al. 2012).

11.4.2.3 The Godavari and Krishna Mangrove Ecologies

In Andhra Pradesh, the Krishna-Godavari mangrove ($16^{\circ}23'N$ and $82^{\circ}20'E$) occupy about 3,3150 ha area (Table 11.2, Fig. 11.1) (Ruban and Gunaseelan 2011) and the largest mangrove i.e., Coringa ($16^{\circ}44'-16^{\circ}53'N$ and $82^{\circ}14'-82^{\circ}22'E$) is situated in the estuary of the Coringa-Gaderu rivers and the Gautami-Godavari distributaries, and receive neritic water from the Bay of Bengal.

11.4.2.4 The Pichavaram and Muthupet Mangrove Ecologies

The Pichavaram mangrove (Fig. 11.1, Table 11.2) forests soil is sandy mud, lie between the Vellar and Coleroon estuaries ($11^{\circ}23'N$ and $79^{\circ}47'E$) in Tamil Nadu supported by tidal neritic water of the Bay of Bengal through Chinnavaikkal river mouth, brackish water from the Vellar and Coleroon and fresh water from the Khan Sahib canal (Fig. 11.1).

The Muthupet mangroves are located ($10^{\circ}15'N$ and $79^{\circ}30'E$) in the southern most end of the Cauvery delta covering 68.03 sq. km (6800 ha) area and 11 km² lagoon on the East coast of India and form a part of the Great Vedaranyam swamp (Table 11.2, Fig. 11.1) (Ashokkumar et al. 2011; Lakshmanan and Selvam 2011; Govindasamy et al. 2012; Srivastava et al. 2012). The soil contains total N 3.70–8.96 $\mu\text{g/g}$, P 0.947–2.827 $\mu\text{g/g}$ and OC 3.721–6.576 mg/g (Ashokkumar et al. 2011).

11.4.2.5 The Cochin Mangrove Ecology

The Cochin mangrove (Fig. 11.1, Table 11.2) soil in Kerala is silty, alluvial, lateritic and sandy. It is one of the largest estuaries (Kadalundi) on the Kerala coast ($10^{\circ}27'N$ and $76^{\circ}15'E$) covering 28 ha mangrove and the Kavvai-Kunhimangalam backwater system occupying 931 ha mangrove forests (Nambiar and Raveendran 2009).

11.4.2.6 The Kundapura Mangrove Ecology

In Karnataka, the poorly known Kundapura mangrove ($13^{\circ}37'24''N$ and $74^{\circ}41'30''E$) forest spreads on about 105 km coastal stretches from Shiroor to Mulky on about 6000 ha area in Udupi district and some smaller mangroves are present in the Dakshina Kannada and Uttara Kannada districts (Table 11.2, Fig. 11.1).

11.4.2.7 The Goa Mangrove Ecology

The Chorao, Mercas, St. Cruz, Panjim, Ribandar and Banastari mangroves of Goa are located at $15^{\circ}00'-15^{\circ}52'N$ to $73^{\circ}30'-74^{\circ}44'E$ on about 120 km coast line on the estuaries of the major rivers i.e., Mandovi-Zuari estuarine complex of ~2000 ha,

Zuari estuary of ~900 ha, Mandovi estuary of ~700 ha and Cumbarjua canal of ~200 ha (Fig. 11.1, Table 11.2). The mangrove soil of Goa is silty sand and silty clay, contain 10–15% salt and pH 8–8.2, although, at five distant locations it was observed that generally alkaline pH vary from 7.79–8.11 (Nayak et al. 2012; Surve et al. 2012).

11.4.2.8 The Ratnagiri Mangrove Ecology

On the West coast of India, the Ratnagiri mangrove is bound by the Arabian Sea at 16°15'00"–17°05'00"N and 73°15'30"–73°22'30"E extending on 0.36 km² area in the Sakhartar estuary in Maharashtra (Fig. 11.1, Table 11.2). Soil of Ratnagiri is laterite, reddish/yellowish grey, clayey-loam to loam texture, pH 4–8 (mostly 7–7.5), total soluble salt 1–3%, total salt 2–38%, OC 0.5–1.5%, total N 0.00–0.09%, available P₂O₅ 10–15 µg/l and available K₂O 10–15 µg/l, although, at a different location, the soil characters were recorded different like phosphate-P 0.30–1.10 µg/l, nitrate-N 1.05–5.03 µg/l, pH 7.4–8.4, salinity 2–38% (Lagade et al. 2011).

11.4.2.9 The Gulf of Kutch and Combay Mangrove Ecologies

The Gulf of Kutch in Gujarat lies between 22–23°N and 68–70° 30'E with an area of approximately 7300 km² (Fig. 11.1, Table 11.2) (Kumar and Ramanathan 2013). The Jakhau–Babber (23°13'59.2"N–68°36'38.1"E), Sangi–Kharo (23°17'36.4"N–68°31'21"E) and Medi–Sinthodi (23°27'54.8"N–68°29'15.1"E) creeks are important constituents of the Kutch (Kachchh or Kachh) mangroves (Saravanakumar et al. 2008, Kumar and Ramanathan 2013).

Although differences of physicochemical properties differed location-wise, the Gulf of Kutch has temperature 18.4–37°C, black and alluvial, silty loam or silty clayey loam soil with pH 5.5–9.0, NaCl balance 32–37%, EC 4.71–6.31 ms/cm, nitrate (0.23–7.26 µM), nitrite (0.04–0.87 µM), phosphate (0.13–3.12 µM), CO₃²⁻ 0.013–0.087%, HCO₃⁻ 0.24–0.91%, Cl⁻ 2.85–2.93%, CaCO₃ 79–85%, available P 0.102–0.222 µg/g, available S 37.70–41.70 ppm, available N 0.031–0.033% (Dudhagara et al. 2008), reactive silicate (4.23–19.02 µM), total organic carbon 0.29–2.56%, inorganic phosphorus 0.12–1.97 mg/g and total N 0.02–1.95 mg/g in different creeks (Saravanakumar et al. 2008). Physico-chemical properties of the soil of Great and Little Rann of Kutch were pH 6.9, 8.1; Na⁺ 46, 65; K⁺ 2, 2.8; Mg²⁺ 16.1, 15.8; Ca²⁺ trace, Cl⁻ 157, 119; SO₄²⁻, respectively (Koyani et al. 2009; Thomas et al. 2012).

11.4.2.10 The Andaman and Nicobar Islands Mangrove Ecology

The Andaman and Nicobar group of Islands lie in the Bay of Bengal (6–14°N and 92–94°E) above 0 to 365 m sea level comprising 572 islands covering about

825,000 ha area on about 1962 km coastline (Fig. 11.1, Table 11.2). The mangrove area is about 617 sq. km which, however, is decreasing over the years. The Island has clayey or sandy loam and acid sulphate soil with pH 4.5–6.82, salinity 6.81 mS/cm, available P 30.00 kg/ha, available K 338.00 kg/ha, total C 0.49% and total N 0.04% (Das 1999; Gopal and Chauhan 2006; Das and Dangar 2008; Das et al. 2011, 2012).

11.5 Salt Lakes

In India, there are 14 well known salt lakes (soda lakes), out of which, 12 lakes are in Ladakh (J and K) on the Himalayas, one (Lonar Lake, a Crater Lake) in Maharashtra and one (Sambhar Salt Lake) in Rajasthan (Table 11.3, Figs. 11.2 and 11.3). Despite the physico-chemical properties of some of the lakes have been worked out (Table 11.3) but to date scanty information are available about microbiology of only the Lonar Lake and Sambhar Salt Lake (Kanekar et al. 2008). The Sambhar Lake encompasses 230 sq. km area, located at 75°05'E and 26°58'N near Jaipur in Rajasthan, known as Salt Lake of Earth which is the intermittent, saline and alkaline lake (Upasani 2008). Whereas, the Lonar Lake has 1.2 km (3900 ft) dia. at about 137 m below the crater rim situated in Buldhana district (19°58'N and 76°31'E) in Maharashtra which was created due to meteorite impact about 50–60 thousand years ago (Kanekar et al. 2008; Hingole and Pathak 2013). The pH of the Lonar lake sediments is 9.27–9.87, alkalinity (CaCO_3) 1.5–1.65 g/kg and salinity i.e., Cl^- 27.50–28.30 g/kg and NaCl 45.38–47.78 g/kg (Kanekar et al. 2008).

Table 11.3 pH and salinity the soda lakes in India

Name	Location	pH	Salinity (%)
Lonar Lake (Crater Lake)	Maharashtra, India	9.5–10.5	1
Sambhar Salt Lake	Rajasthan, India	9.5	7
Khyagar Lake	Ladakh (J & K), India	9.5	0.6
Tso Moriri Salt Lake	Ladakh (J & K), India	9.0	NA
Tso Kar Salt Lake	Ladakh (J & K), India	8.8	NA
Lake Surigh Yilganing Kol	Ladakh (J & K), Aksai Chin, India	NA	NA
Tso Tang Lake	Ladakh (J & K), Aksai Chin, India	NA	NA
Aksayqin Hu Lake	Ladakh (J & K), Aksai Chin, India	NA	NA
Lake Hongshan Hu	Ladakh (J & K), Aksai Chin, India	NA	NA
Tianshuihai lake	Ladakh (J & K), Aksai Chin, India	NA	NA
North Tianshuihai lake	Ladakh (J & K), Aksai Chin, India	NA	NA
Kushul lake	Ladakh (J & K), Aksai Chin, India	NA	NA
Pangong Salt Lake	Ladakh (J & K), India and China	9.4	0.9
Spanggur Tso (Pongur Tso)	Ladakh (J & K), India and China	NA	NA

NA not available

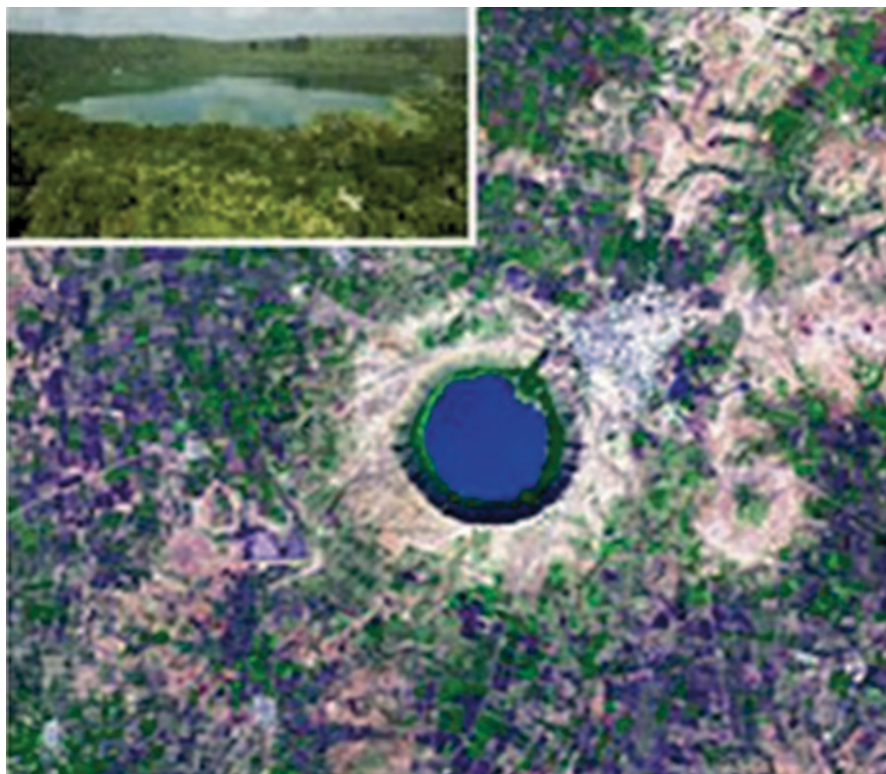


Fig. 11.2 Lonar crater lake. Inset—closer view

11.6 The Coastal Saline Ecology

India has a very long coast line of about 2700 km. In the eastern side is the coast of Bay of Bengal spreading from West Bengal to Kanyakumari and in the western side is the coast of the Arabian Sea spreading from Kanyakumari to the Runn of Kutch in Gujarat (Fig. 11.1, Table 11.1). Wide climatological differences along the two coastlines have observed very higher degree variations of the biotic and abiotic conditions. However, physico-chemical characters and microbiology have been studied in discrete coastal locations only. The physico-chemical properties of East and West coastal soils of India are highly variable and do not have any relation with soil type (Tripathi et al. 2007; Das and Dangar 2008; Barua et al. 2011; Bal et al. 2012; Lakshman et al. 2013) (Table 11.4). In the two coastal locations viz., Canning (WB) of East coast and Calicut (Kerala) of West coast, the ion balance (me/l) viz., Na^{2+} 156 and 320, Ca^{2+} 29 and 30, Mg^{2+} 120 and 93, Cl^{-} 213 and 354, SO_4^{2-} 16 and 102, respectively and sodium adsorption ratio (SAR) i.e., 18 and 41, respec-



Fig. 11.3 Sambhar lake. (Upasani 2008; NASA: ISSOI0-E-85245)

tively were variable, and in coastal Gujarat, sulfur contents vary from 0.11–0.28% (Yousuf et al. 2012).

11.7 The Inland Saline Ecology

In India, nearly 9.38 m ha area has salt-affected soils, out of which 5.5 m ha is saline (including coastal soil) and 3.88 m ha is alkali soil (IAB 2000)(Table 11.1, Fig. 11.1). Inland salinity is mainly the result of over exploitation ground water for agricultural and industrial purposes. The inland saline area in India covers the arid and semiarid regions of Rajasthan, Haryana, Punjab, Gujarat, Uttar Pradesh, Delhi, Andhra Pradesh, Maharashtra, Karnataka and Tamil Nadu. The soils ($n=26$) of Haryana are categorized as non-saline soils (EC 0–2 dS/m), weakly saline soils (EC 2–4 dS/m), saline soils (EC 4–8 dS/m), strongly saline soils (EC 8–16 dS/m) and very strongly saline soils (EC > 16 dS/m). The saline soils show electrical conductivity (EC) range 1.04–21.00 dS/m, pH 6.03 to 8.62, while organic C, total N, and available P are in the range of 0.06–0.94%. Whereas, the characters of the

Table 11.4 Soil physico-chemical properties of coastal India

Location	pH	Salinity (mS/cm)	Soil type	Available P (kg/ha)	Available K (kg/ha)	Total C (%)	Total N (%)
Sundarbans coast (NM)	5.64– 6.94	5.74–11.32	Sandy loam	10–20	87.6–311.5	0.061– 0.085	0.567– 0.839
Namkhana, WB	5.71	7.71	Clayey	18.00	575.00	0.18	0.13
Canning, WB	4.99– 7.28	2.02–19.58	Sandy loam	NA	NA	0.6–1.0	0.06– 0.10
Dhunchi, WB	5.82	7.61		18.00	425.00	0.27	0.05
Kakdweep, WB	4.63	7.52	Clayey	18.00	325.00	0.21	0.05
Ramaganga, WB	4.71	6.93	Clayey	15.00	263.00	0.21	0.30
Susunia, WB	4.81	6.54	Clayey	24.00	775.00	0.42	0.14
CRRI, Orissa	6.42	5.32	loam	22.00	102.00	0.45	0.06
Chilka, Orissa	6.42	8.53	Sandy loam	16.00	612.78	0.43	0.25
Garakujanga, Orissa	5.62– 6.0	4.62–8.61	Sandy loam	84.34	688.00	0.45	0.09
Bhuiyapada, Orissa	6.62	8.61	Sandy	30.00	173.00	0.35	0.06
Ersama, Orissa	6.72	8.81	Clayey	18.00	367.01	0.32	0.23
Port Blair, AN Islands	6.82	6.81	Sandy	30.00	338.00	0.49	0.04
Mahe, Pudhucherry	6.41	5.23	Sandy	27.00	113.00	0.15	0.05
Calicut, Kerala	4.4	5.64	Sandy loam	NA	NA	0.48	NA
Mangalore, Karnataka	6.52	7.12	Clayey	21.00	300.00	0.49	0.03
Kumta, Karnataka	5.56	6.84	NA	24.4	288.67	0.27	NA
Costal Gujarat	8–8.3	3.8–7.1	NA	NA	NA	1.27–1.38	0.09

Data from rice soils but Kumta and Gujarat

NM non-mangrove, NA not available

sandy soil of Nayabans, Haryana has pH 7.2, CEC 8.4 me/100 g, EC 78 mS/cm, OC 0.8%, Na^{2+} 692 me/l, Ca^{2+} 22 me/l, Mg^{2+} 590 me/l, Cl^{-} 1360 me/l, SO_4^{2-} 75 me/l and SAR 36.

The Kharaghoda (Gujarat) soil temperature changes between 29–31 °C and pH between 7.9 and 9.1 (Vora and Modi 2013). In West Bengal, inland saline area is very limited. Bhattacharyya et al. (2013) observed that at Sagar, saline soil is clayey, pH 5.3, OC 0.11 % and CEC 18.5.

In India, inland saline soils vary in different districts viz., Varanasi, Mau, Ballia and Ghazipur of Uttar Pradesh, India are whitish, sandy loam with ECE 10.47–5.69 dS/m, pH 8.0–9.5, organic C 0.27–2.29%, Varanasi region contain maximum N and K_2O (219.5–159.3 kg/ha), while Ballia district possess maximum PO (13.34 kg/ha) (Upadhyay et al. 2012). Furthermore, the inland sodic soils of five

Table 11.5 Classification of halophilic microorganisms

Types of microorganisms	Salt (NaCl) (%)	Concentration (M)
Slightly halophiles	2–5	0.3–0.8
Moderate halophiles	5–20	0.8–3.4
Extreme halophiles	>20	>3.4

locations at Kasrawa, Hardoi, Thakurenda, Paschim Gau and Gurbakshganj in Rae Bareilly district of Uttar Pradesh has pH 9.65–10.2 and EC 0.75–4.55 (Table 11.4) (Damodaran et al. 2013).

11.8 Classification of Microbes in Response to Salt Concentration

In general, the organisms tolerating >4% NaCl is considered as salt stress tolerant. According to response of the microbes to salt concentration, they are grouped as halotolerant and halophilic organisms. The former group show some degree of salt (NaCl) tolerance, mostly have upper and lower limits but the true salt tolerant (euryhaline) microorganisms adapt to any salt concentration (0.05 to 5.5 M saturation point) depending on their intrinsic character. The halophilic bacteria denotes “salt loving” or in microbiological terms, “salt requiring” and grouped as slight halophiles (intestinal, rumen and marine bacteria) requiring about 0.3 M (2.0%) to 0.8 M (5.0%) NaCl, moderate halophiles requiring 0.8 to 3.4 M (20%) NaCl and extreme halophiles requiring more than 3.4 M (20%) NaCl (Ventosa et al. 1998; Ventosa 2004). However, it may be advantageous to differentiate them as facultative (no compulsory requirement) halophiles and euryhaline (halotolerant) bacteria. Both halophilic and halotolerant microorganisms are found in saline soils probably, because they can adapt to grow in high salt concentration. Halophilic microbes are classified as slight halophiles that grow optimally in 3% (w/v) total salt, moderate halophiles with optimal growth at 3–15% (w/v) salt and extreme halophiles that grow optimally at 25% (w/v) salt (Ventosa et al. 1998; Ventosa 2004) (Table 11.5). The detailed microbiology, biochemical and molecular adaptation has been described elsewhere (Dubey and Maheshwari 2012).

11.8.1 Osmoregulation of Microbes

The bacteria, algae and plants accumulate osmotic solutes (osmolytes) viz., carbohydrates, amino acids viz., proline and their different derivatives viz., betaines (Verma 1993) and regulate the enzymes viz., super oxide dismutase, catalase, peroxidase, polyphenol oxidase, glycine betaine synthetase etc. (Csonka 1989; Csonka and Hanson 1991; Grene 2002; Moat et al. 2002; Das and Dangar 2008) positively with stress.

Cellular K⁺ content and NaCl tolerance are distinctly correlated with osmotic stress regulation. The non-halophilic bacteria acquire very little K⁺, thereby un-

able to tolerate $>2\%$ NaCl in medium, extreme halophiles (*Halococcus morrhuae* and *Halobacterium cutirubrum*) can grow with 4.25 M NaCl and cytoplasm also accumulates 4.25 M K^+ as KCl, the salt tolerant bacteria (*Staphylococcus aureus*) grow over a wide (0–2.5 M) range of NaCl and possess virtually equal (20–100 M/100 mg dr. wt.) amount of cytoplasmic KCl (Csonka 1989; Csonka and Hanson 1991; Grene 2002; Moat et al. 2002).

In general, algae and yeast concentrate on K^+ and polyols (glycerol and arabinitol) as compatible solutes which may act both as an osmoregulator to maintain cellular osmotic balance and protect enzymatic function but the bacteria prefer K^+ , amino acids, betaine etc. as a compatible solute (Verma 1993; Moat et al. 2002). Most non-halophilic organisms respond to sudden NaCl increase by losing some cell water which results in an effective K^+ increase (Csonka 1989; Csonka and Hanson 1991; Vreeland 1993; Grene 2002; Moat et al. 2002, Yadav and Yadav 2003).

11.8.2 Molecular Basis of Osmoregulation of Osmotic Stress Tolerance in Microbes

E. coli and other enteric bacteria have evolved genes that are osmotically regulated at the transcriptional level for uptake and synthesis of specific osmolytes (Vreeland 1993; Yadav and Yadav 2003). Proline, glycine betaine, choline etc. have been shown to stimulate bacterial growth and nitrogen fixation (*Klebsiella pneumoniae*) when added to media of high osmotic strength (Csonka 1989). Thus, proline overproduction clearly increases osmotolerance in bacteria (Csonka 1989; Csonka and Hanson 1991; Verma 1993; Yadav and Yadav 2003).

11.9 Microbiology and Plant Growth Promotion of Saline Soils

Salinity affects microbes, agricultural crops, biodiversity and environment and only the salt tolerant (osmotolerant or halotolerant) plant growth promoting rhizobacteria (PGPR) that can contribute to improve soil and plant health in stressed environment. Therefore, idea of osmotolerance mechanisms of these PGPRs have great importance for maintenance of soil health and sustenance of plant growth (Yadav and Yadav 2003; Tripathi et al. 2007; Maheshwari 2011, 2012, 2013). Besides, as response of microbes to different stresses is similar, osmotolerant microbes would endure other viz., temperature, drought, oxic-anoxic stresses etc. also and maintain ecological functions. However, root colonization potential of the PGPRs is not known to be hampered by higher salinity (Paul and Nair 2008).

Halotolerant PGPRs can alleviate salt stress and maintain growth of plants. For example, *Brachybacterium saurshtrense*, *Brevibacterium casei* and *Haererohalobacter* sp. could enhance growth of *Arachis hypogaea* under salt stress (Shukla et al. 2011), salinity (6% NaCl) tolerant PGPRs of tomato could maintain the PGP func-

tions like phosphate solubilization (25 mM), produce siderophore (1000 µg/ml and ACC deaminase (50 µM/mg/h) and enhance up to 50% growth over NaCl stress (Tank and Saraf 2010), and biofertilizer (*Azotobacter* and *Azospirillum* and different other microbes) application in saline and iron toxic fields enhanced 3.8–109% rice production (Yanni et al. 2011; Sahoo et al. 2013a, b, c). However, nitrogenase activity is greatly affected by salt stress in species of *Azospirillum*, *Klebsiella*, and *Azotobacter* etc. however, it was observed that their phytohormone (IAA) production remained unaffected. To adapt to soil salinity/osmolarity, *Azospirillum* sp. accumulate compatible solutes viz., glutamate, proline, glycine betaine, trehalose etc. by either synthesis or accumulation wherein proline play major role in osmoadaptation of *A. brasilense*. As rice and maize are unable to synthesize glycine betaine (GB), they would benefit by rhizospheric colonization of non-GB requiring PGPRs. Nevertheless, different organisms use varying types of chemicals for osmotolerance and the organisms may shift osmolite metabolism with change of stress level (Tripathi et al. 1998). Dudhagara et al. (2008) observed that the P solubilizing bacteria can grow at 25–45 °C, slightly alkaline pH (6–10), tolerate 2.5–10% NaCl and fix nitrogen and the N₂ fixers could tolerate 10% NaCl but optimum is at 2.5% proving that these PGPRs could be effective biofertilizers in saline conditions.

11.9.1 Microbial Dynamics of Mangrove Ecosystem of India

Saline habitat of mangrove ecosystems harbors the Archaea and Eubacteria. The Archaea are extremely halophilic (about 30% NaCl tolerance) primary decomposer microbes (Zahran 1997) which utilize low concentration of dissolved organic compounds, assimilate inorganic substances like nitrate and phosphate, decompose nutrient-poor plant tissues and have efficient anaerobic metabolism i.e., adapted to both aerobic and anaerobic status (Holguin et al. 2001; Das et al. 2005, 2011, 2012). Various groups of bacteria like nitrogen fixers, phosphate solubilizers, cellulose decomposers, nitrifiers and denitrifiers, sulphur and iron oxidizers, iron reducers, methanogens, magnetic behavioral, human pathogens, antibiotics and enzyme producer etc. are usually present in the ecosystem (Holguin et al. 2001; Das et al. 2005, 2011, 2012).

The roots of the mangrove plants bind soil and help in establishment of the microorganisms which stabilize the native ecology. Diverse microbial communities are the food source of other organisms, resources of the nutrients and metabolites of diverse utility (Holguin et al. 2001; Sahoo and Dhal 2009; Mishra et al. 2011; 2012a, b). The plant growth-promoting bacteria (PGPB), besides improving crop yield, solve environmental problems viz., soil erosion, restoration of mangrove ecosystems, phytoremediation etc. (Holguin et al. 2001; Das et al. 2005, 2011, 2012; de-Bashan et al. 2012). The microbes in the mangrove ecosystems also transform nutrients from dead mangrove vegetation into N, P and other mineral nutrients that are used by the plants and use the root exudates for their growth (Holguin et al. 2001; Sahoo and Dhal 2009; Mishra et al. 2011, 2012a, b). Investigations showed that application of the diazotrophic PGPB i.e., cyanobacterium *Microcoleus chthonoplastes* increased root colonization of black mangroves and N accumulation

Table 11.6 Microbial population of mangrove ecosystem

Mangrove	Cellulose degrad- ing bacteria	Phosphate solu- bilizing bacteria	Nitrogen fixing bacteria	Sulphur oxi- dizing bacteria
Bhitarkanika (cfu $\times 10^5$ /g)	2.50–8.77	7.32–10.51	113.09–224.17	14.13–22.55
Sundarbans (cfu $\times 10^6$ /g)	4.756–6.189	0.584–0.760	0.387–4.481	0.236–0.415
Gulf of Kutch (cfu $\times 10^3$ /g)	5–13	3–17	4.0	NA

NA not available

in plants; several species of *Azotobacter brasilense*, *A. chroococcum*, *A. vinelandii*, and *A. beijerinckii* and *Azospirillum halopraeferens* could enhance growth of different mangrove seedlings; *Oceanobacillus picturae* efficiently solubilize phosphate in mangroves and many bacteria (including PGPB) in *en mass* can remediate soil (de-Bashan et al. 2012). Functionality of the microbes viz., nitrogen fixing bacteria (*Azospirillum brasilense*, *Azotobacter*, *Rhizobium*, *Clostridium*, *Klebsiella*, *Listonella*, *Phyllobacterium* etc.) of Pichavaram, Sundarbans, Bhitarkanika; sulfate reducing bacteria in Goa; purple photosynthetic bacteria studied in Pichavaram; iron oxidizing/reducing bacteria in Goa and Konkan; methanogens in Pichavaram and Kodiakkarai; anaerobic and microaerobic photosynthetic bacteria and actinobacteria in Cochin; sediment fungi in Goa, Andaman-Nicobar, Pichavaram, Cochin, Alibag mangrove sediments and P solubilizing microbes (*Bacillus amyloliquefaciens*, *B. atrophaeus*, *Paenibacillus macerans*, *Xanthobacter agilis*, *Vibrio proteolyticus*, *Enterobacter aerogenes*, *E. taylorae*, *T. asburiae*, *Kluyvera cryocrescens*, *B. licheniformis*, *Chryseomonas luteola*, *Pseudomonas stutzeri* etc.) associated with mangroves have been well recognized (Kumar 2000; Holguin et al. 2001; Sahoo and Dhal 2009; Mishra et al. 2011, 2012a, b). As mangrove sediments are mainly anaerobic with a thin aerobic surface layer, the sulfate reducing bacteria (*Desulfovibrio desulfuricans*, *Desulfotomaculum orientis*, *Desulfococcus multivorans* etc.) are mainly involved in CO₂ emission i.e., organic decomposition (viz., propionate, butyrate, benzoate etc.), P and Fe amelioration (Holguin et al. 2001; Sahoo and Dhal 2009). On the other hand, methanogens (*Methanococcoides methylutens* and unidentified thermo-tolerant methanogens) fluctuate between 3.6×10^2 – 1.1×10^5 cfu/g wt. sediment depending on soil salinity and other physical characters which are important microbial guilds of mangroves and are dependent on the S-reducing bacteria (Holguin et al. 2001; Sahoo and Dhal 2009), whereas, dynamics of other microbial guilds of some mangroves vary between 10^3 – 10^6 cfu/g soil (Table 11.6).

Microbial diversity viz., algal diversity and N₂-fixing bacteria in the Sunderbans, West Bengal, fungal diversity of mangroves of Pichavaram in Tamilnadu, Maharashtra coast and southwest coast, P-solubilizer in Vellar estuary at Parangipettai, sulfate reducers in Goa have been reported but knowledge on mangrove microbial functionality is very poor (Kumar 2000; Mishra et al. 2012a, b).

11.9.2 Microbes of the Bhitarkanika and Mahanadi Delta Mangrove

Microbial guilds of Bhitarkanika have been studied by the present authors at sparse to dense (core) mangrove sites (Table 11.6). At the Rangani, Mahisamunda, Habalaganda and Kalibhanjadian mangrove sites, the population ($\times 10^5$ cfu/g soil) of phosphate solubilizers (7.32–10.51), sulphur oxidizers (14.13–22.55), free living N_2 fixers (113.09–224.17) and cellulose degraders (2.50–8.77) were recorded. Out of them, 27 isolates were efficient degraders viz., *Pseudomonas* sp. ($n=4$), *Paenibacillus polymyxa*, *B. mycoides*, *B. brevis* were cellulose degraders, *Pseudomonas* sp. ($n=2$), *P. cepacia*, *P. stutzeri*, *B. lichiniformis*, *B. schlegelii* and *Bacillus* sp. solubilized phosphate; *Klebsiella* sp., *Azotobacter* sp., *Bacillus* sp., *B. alcalophilus*, *Pseudomonas* sp. and *P. putida* fixed N_2 asymbiotically; *Desulfotomaculum* sp. ($n=3$), *Desulfomonas* sp., *Desulfovibrio* sp., *D. salexigens* and *Pseudomonas* sp. oxidized sulphur which tolerated $\geq 10\%$ or more NaCl (Mishra et al. 2009, 2011, 2012a, b). From the sediment, 27 fungi viz., *Acremonium byssoides*, *Alternaria alternata*, *Aspergillus flavus*, *A. niger*, *A. oryzae*, *Choenophora cucurbitarum*, *Cladosporium oxysporum*, *Curvularia lunata*, *Drechslera hawaiiensis*, *Fusarium chlamydosporum*, *F. moniliforme*, *F. oxysporum*, *F. redolense*, *F. solani*, *Neocosmospora vasinfectum*, *Neosortaria fischeri*, *Paecilomyces lilacinous*, *P. varioti*, *Phoma glomerta*, *Penicillium digitatum*, *P. citrinum*, *P. oxalicum*, *Rhizopus stolonifer*, *Trichoderma harzianum*, *T. viride* and *Byssoschlarnus niveus* were identified. Survey revealed occurrence of 11 algal species such as *Spirogyra* sp., *Anabaena* sp., *Gloeocapsa* sp., *Oscillatoria* sp., *Chlorella* sp., *Ulva* sp., *Chlorococcus* sp., *Chlamydomonas* sp., *Phormidium* sp., *Microcystis* sp. and *Lyngbya* (*L. cylanica* and *L. semiplena*) sp. in the Bhitarkanika mangrove (Mishra et al. 2009, 2011, 2012a, b; Thatoi et al. 2012). Bhitarkanika contains large amount of C and other nutrients which support different microbial communities responsible for major nutrient transformations (Holguin et al. 2001; Mishra et al. 2009; 2011, 2012a, b) and supported report of Zahran et al. (1992) that the Gram-positive bacteria are well represented in the saline habitat and the *Bacillus* sp. and *Micrococcus* sp. are mimetically dominant. The fungal genotypes vary widely in the Bhitarkanika mangrove ecology viz., *Rhizopus stolonifer* predominates (378×10^5 cfu/g soil) followed by *Fusarium solani* (351×10^5 cfu/g soil), *Aspergillus oryzae* (292×10^5 cfu/g soil), *Alternaria alternata* (180×10^5 cfu/g soil) and *Fusarium oxysporum* (178×10^5 cfu/g soil) in different sites (Thatoi et al. 2012). Behera et al. (2012) opined that mangrove fungi constitute the second largest ecological group, recorded 22 fungal genotypes of 13 genera belonging to Ascomycetes and one genus of Actinobacteria. Population ($\times 10^4$ cfu/g soil) of *A. alternata* was highest 23.00, followed by *A. oryzae* (22.00) and *Phoma* sp. (22.00), and *F. oxysporum*, *Verticillium* sp., *P. citrinum*, *P. sclerotium* and *T. viride* were not found in coastal soil (Behera et al. 2012). However, overall microbial pool ($\times 10^5$ cfu/g soil) in soil decline in the sequence of heterotrophic, free living N_2 fixing, Gram-negative, nitrifying, sulphur oxidizing, Gram-positive, spore forming, denitrifying, anaerobic, phosphate solubilizing, cellulose degrading bacteria, fungi and actinomycetes. The *Bacillus*, *Pseudomonas*, *Desulfotomaculum*, *Desulfovibrio*, *Desulfomonas*, *Methylococcus*, *Vibrio*, *Micrococcus*, *Klebsiella* and *Azotobacter*

were predominant bacterial genera (Mishra et al. 2012a, b). In the Bhitarkanika mangrove soils, the Gram negative bacteria more and predominant ones were *Pseudomonas aeruginosa* and *P. alcaligenes*, *Methylococcus* sp. and *Desulfotomaculum* sp. (Mishra et al. 2009, 2012a, b).

11.9.3 Bacterial Flora of the Sundarbans

The diversity of bacteria and fungi in the Sundarbans have not been examined thoroughly (Table 11.6), although some reports mention various microorganisms in the soils and on decomposing litter, besides pathogens infesting mangrove leaves, fish, prawns and mammals (Das 1999; Gopal and Chauhan 2006; Das et al 2011, 2012). Total bacteria ($7.65\text{--}14.5 \times 10^4$ cfu/g soil) varied widely at different locations of the mangrove and had positive correlation only with total nitrogen content in soil i.e., total nitrogen is the driving factor of bacterial communities in the mangrove (Hossain et al. 2012). Different authors have identified the *Brevibacterium lypolyticum*, *B. marinopiscosum*, *B. minutiferula*, *B. sociovivum*, *Clostridium carnis*, *C. pectinivorum*, *Lactobacillus brevis*, *L. delbrueckii*, *Listeria monocytogenes* and *Micrococcus agilis* and the fungi like *Aspergillus*, *Collectotrichium*, *Fusarium* and *Helminthosporium* sp. as common inhabitants of the mangrove (Das 1999; Das et al 2011, 2012; Gopal and Chauhan 2006). Cellulose decomposers (45.15×10^4 cfu/g) prevailed in this mangrove in comparison to P solubilizing, N_2 fixing and S oxidizing bacteria (Ramanathan et al. 2008). Among eight different bacterial phyla viz., Alpha-, Beta-, Gamma- and Delta- Proteobacteria, Flexibacteria (CFB group), Actinobacteria, Acidobacteria, Chloroflexi, Firmicutes, Planctomycetes and Gammatimonadates where the γ -Proteobacteria were most abundant in Sundarban sediment (Ghosh et al. 2010). *Bacillus cereus*, a salt-tolerant PGPR possesses IAA, protease, chitinase, siderophore production, P solubilization, and superoxide dismutase, peroxidase, ascorbate peroxidase and catalase activities enhanced growth and salt tolerance of *Vigna*, *Cicer* and *Oryza* species (Chakraborty et al. 2011).

11.9.4 Bacterial Flora of the Pichavaram and Muthupet

It has been observed that, salinity has negative correlation but pH and dissolved oxygen showed no significant influence on the mycoflora. The ratio between fungal and bacterial populations is about 1:7000 in these mangrove sediments and bacterial population is more in sediment (Ravikumar 1995; Ravikumar and Vittal 1996; Behera et al. 2012). The *Vibrio*, *Bacillus*, *Micrococcus*, *Pseudomonas*, *Aeromonas*, *Flavobacterium*, *Azotobacter* (n=3) sp. etc. and the magnetobacteria viz., *Pseudomonas mesophilica*, *P. caryophyllis* and *B. cereus* prevail in these mangroves (Ravikumar 1995; Ravikumar and Vittal 1996; Sahoo and Dhal 2009). *Azotobacter* sp. viz., *A. vinelandii*, *A. beijerinckii* and *A. chroococcum* are more common in mangrove sediments than in marine, backwater and estuarine systems (Lakshmanaperumalsamy 1987). A broad-spectrum antibiotic, the unsaturated lactone,

produced by *Streptomyces griseobrunneus* having wide range antimicrobial activity has been isolated from this ecology (Ravikumar 1995; Ravikumar and Vittal 1996; Sahoo and Dhal 2009).

From Muthupet lagoon, 17 cyanobacterial species viz., *Microcystis robusta*, *Synechocystis* sp., *Gloeocapsa* sp., *Aphanocapsa koordersi*, *A. bulolosa*, *A. littoralis*, *Johannesbaotistia pellucida*, *Phormidium* sp., *Spirulina major*, *Porphyrosiphon natarsii*, *Schizothrix telephorides*, *Oscillatoria claricentrosa*, *O. vizagapatensis*, *O. tenuis*, *O. curviceps*, *O. calcuttensis* and *O. claviceps* were recorded (Selvakumar and Sundararaman 2001).

In Muthupet mangroves total heterotrophic bacteria count $43\text{--}77 \times 10^{-6}$ cfu/g dr. wt. (Ashokkumar et al. 2011) was more during pre-monsoon season and decreased during summer season, and the bacteria were more in sediment than water. From sediment, 32 species of bacteria belonging to *Bacillus*, *Carnyobacterium*, *Vibrio*, *Micrococcus*, *Azotobacter*, *Azospirillum*, *Phosphobacteria* and *Pseudomonas* were characterized which fluctuated between 144×10^3 to 175×10^4 cfu/g sediment at different locations and the halophilic bacteria fluctuated from 120×10^2 to 663×10^6 cfu/g sediment and they increased during summer when salinity was also high. High inflow of fresh water i.e., decline of salinity and higher predation of microbes at bottom level would reduce halophiles in monsoon (Govindasamy et al. 2012). Among the fungi, *Penicillium*, *Aspergillus* etc. were reported from the Muthupet mangrove soil (Ashokkumar et al. 2011; Govindasamy et al. 2012).

The microbes are versatile PGPRs as all of them possess IAA and catalase activities and 95% produced ammonia (Ashokkumar et al. 2011; Srivastava et al. 2012; Govindasamy et al. 2012). Although variable i.e., tolerance of the heavy metals like Fe, Zn, Pb, Mg and Cu by *Bacillus*, *Pseudomonas*, *Aspergillus* and *Penicillium* sp. but lesser or in-tolerance of the *Azotobacter*, *Azospirillum*, *Phosphobacteria* and *Glucanacetobacter* sp. proved that a large pool of microbes would be effective PGPR under stress conditions (Ashokkumar et al. 2011; Srivastava et al. 2012; Govindasamy et al. 2012).

11.9.5 Microbes of Goa Mangroves

Microbial information from Goa mangrove is scanty. Microbial dynamics of alkaliphilic bacteria varied between $68.61\text{--}210.33 \times 10^5$ cfu/g soil (Nayak et al. 2012). The sulfate reducing bacteria were well known and eight species viz., *Desulfovibrio desulfuricans*, *D. aestuarii*, *D. selexigens*, *D. sapovorans*, *Desulfotomaculum orientis*, *D. variabilis*, *Desulfococcus multivorans* were recorded which can metabolize a wide range of simple organic compounds viz., propionate, butyrate, benzoate etc. and P and Fe availability depends on SO_4 reducing bacteria in sediment (Sahoo and Dhal 2009).

In central West coast of India viz., Ratnagiri, Mormugao, Karwar and total bacterial count was higher in monsoon ($12\text{--}30 \times 10^5$ cfu/ml) than pre-monsoon ($2.6\text{--}12.2 \times 10^5$ cells/ml) and post-monsoon ($1.8\text{--}9.1 \times 10^5$ cells/ml).

11.9.6 *Microbes of Gulf of Combay, Gulf of Kutch*

In the Gulf of Kutch, total bacterial and fungal load ranged 35–61 cfu/g and 7–20 cfu/g soil, respectively. Population of nitrate forming bacteria was 4.0×10^3 cfu/g soil, phosphate solubilizing bacteria varied from $3\text{--}17 \times 10^3$ cfu/g soil, cellulose degrading bacteria varied from $5\text{--}13 \times 10^3$ cfu/g soils (Table 11.6) (Goutam and Ramanaathan 2013). The 15 bacterial isolates of different locations of saline desert (Kutch) were reported which grew at 6–10 pH and 0–20% w/v salt concentration, tolerated the temperature up to 50°C (Koyani et al. 2009). The population of Archaea in the hypersaline soils of the Great Rann of Kutch ranged from 6.26×10^3 to 3.42×10^4 cfu/ml (0.1% of total population) across locations (Thomas et al. 2012).

11.9.7 *Microbes of the Andaman and Nicobar Mangroves*

Microbial studies in the Andaman and Nicobar mangroves are extremely poor. Meager information shows that in Little Andaman, total heterotrophic bacteria population density varies from $5.2\text{--}273 \times 10^2$ cfu/ml in the water samples and from $6.2\text{--}40 \times 10^3$ cfu/g in the sediment samples (Swarnakumar et al. 2008).

The mangroves of Andaman and Nicobar Islands are inhabited by 17 fungi and *Verruculina enalia* is most prevalent associated with *Acrocordiopsis*, *Aigialus*, *Aniptodera*, *Antennospora*, *Anthostomella*, *Ascocratera*, *Bathyasus*, *Belizeana*, *Biatriospora*, *Caryosporella* sp. etc. (Vittal and Sharma 2006).

11.9.8 *Microbes of the Udyavara Mangrove*

From the Udyavara mangrove on the Udyavara and Katpady river mouths of Karnataka coast, 91 fungi of 68 genera were recovered with higher richness and diversity during monsoon. The *Rhizophora mucronata*, *Bruguiera gymnorrhiza*, *Sonneratia caseolaris*, *Lignincola laevis*, *Savoryella lignicola*, *Trichocladium linderi*, *Paseriniella mangrovei*, *S. paucispora*, *Trichocladium achrasporum* and *T. linderi*, *Cirrenalia pygmea*, *Lulworthia grandispora*, *Nais* sp. and *Zalerion varium* were dominant species (Maria and Sridhar 2003).

11.9.9 *Microbes of the Cochin Mangrove*

Thirty marine fungi were recorded from the Cochin mangrove ecology. Among them, 19 were Ascomycetes, 1 Basidiomycete and 10 Mitosporic fungi viz., *Halocyphina villosa*, *H. minuta*, *H. oceanica*, *Aniptodera chesapeakeensis*, *Lignicola laevis*, *L. longirostris*, *Lulworthia grandispora*, *Verruculinia enalia*, *Periconia prolific*, *Trichocladium achrasporum*, *Halosarpheia*, *Aniptodera chesapeakeensis*, *A. salsuginosa* and *Leptosphaeria australiensis* were found frequently (Nambiar

and Raveendran 2009). In Cochin mangroves, the photosynthetic anoxygenic sulfur and purple sulfur bacteria or the purple non-sulfur bacteria that use H_2S or other reduced inorganic substrates were predominantly *Chromatium*, *Chloronema Beggiotoa*, *Thiopedia*, *Leucothiobacteri*, *Rhodospirillum* and *Rhodopseudomonas* sp. (Sahoo and Dhal 2009).

11.9.10 Microbes of the Krishna-Godavari Mangrove

Microbial population range in Krishna-Godavari mangrove was recorded as 2.0×10^4 – 1.9×10^5 cfu/g soil. The prevalent bacteria are: *Bacillus*, *Actinobacter*, *Enterobacter*, *Legionella*, *Pseudomonas*, *Klebsiella*, *Microbacterium*, *Lactobacillus*, *Clostridium*, *Rhizobium*, *Yersinia*, *Carnybacterium*, *Serratia*, *Proteus*, *Vibrio*, *Morganella*, *Carnybacterium*, *Staphylococcs*, *Moraxella*, *Aeromonas*, *Nesaria*, *Enterococcus*, *Micrococcus* sp., *E. coli* etc. (Ruban and Gunaseelan 2011).

The Godavari-Krishna delta is inhabited different fungi and 11 fungal species were recorded from Godavari and five from Krishna delta regions. In these mangroves, *Verruculina enalia* is most prevalent along with *Chaetomastia*, *Coccostroma*, *Dactylospora*, *Didymella*, *Eutypa*, *Halorosellinia*, *Halosarpheia*, *Hypoxylon*, *Julella*, *Kallichroma*, *Leptosphaeria*, *Masasarina* sp. etc. are common inhabitants (Vittal and Sharma 2006).

11.10 Microbial Dynamics of Coastal Saline Ecosystems of India

The microbial diversity of the East coast and West coast of India viz., Sundarbans coast, Namkhana, Canning, Dhunchi, Kakdwep, Ramaganga, Susunia (WB); CRRRI, Chilka, Garakujanga, Bhuiyapada, Ersama (Orissa), Port Blair (AN Islands); Mahe (Puducherry); Calicut (Kerala); Mangalore, Kumta (Karnataka) and Costal Gujarat was studied relatively more thoroughly and observed that they belonged to all known microbial genotypes (Das and Dangar 2008; Das et al. 2008; Ramanathan et al. 2008; Barua et al. 2011). In the coastal West Bengal, Orissa, Andhra Pradesh, Puducherry and Karnataka, microbial guilds are highly variable and diversity is grossly incoherent of soil physico-chemical constituents. The population (cfu/g soil) ranges were: heterotrophic bacteria 10^6 – 10^7 , spore producers 10^5 – 10^7 , gram negative bacteria 10^5 – 10^7 , nitrifying bacteria 10^5 – 10^6 , denitrifying bacteria 10^4 – 10^5 , phosphate (P) solubilizing bacteria 10^4 – 10^5 , asymbiotic nitrogen fixing bacteria 10^4 – 10^5 , sulphur oxidizing bacteria 10^3 – 10^4 , fungi 10^3 – 10^4 and actinobacteria 10^2 – 10^4 . The *Bacillus thuringiensis* (Bt) and *Pseudomonas* (Ps) population in the soils were structurally and functionally diverse, possessed polyvalent functions, population sizes were 1.44 – 2.40×10^4 cfu/g soil and 0.01 – 0.63×10^6 cfu/g soil, respectively. The PGP functions, insecticidal activity and the crystal morphotype of

Bt and toxicity of Ps populations were highly variable and independent of the habitat (Das and Dangar 2008; Das et al. 2008). The Bt population tolerated 5–12% NaCl and about 98% other organisms tolerated 3–18% NaCl (Das and Dangar 2008; Das et al. 2008). The aerobic heterotrophic and spore forming bacteria were about ten times higher than the anaerobic counterparts (Das and Dangar 2008; Das et al. 2008; Barua et al. 2011). In the Gujarat coast also, bacterial guilds were highly diverse, population ranged $8\text{--}34 \times 10^5$ cfu/g soil and 13 bacterial isolates could grow at 2–12% salt and pH 2–12 (Vora and Modi 2013) and the biosurfactant producing microorganisms of hot springs, ocean, oil wells, petrol pump, desert and costal soils etc. belonged to *B. licheniformis*, *B. subtilis*, *B. polymyxa*, *B. macerans*, *B. pumilus* and *B. coagulans* (Joshi et al. 2013). Molecular analysis revealed that the phylotaxons of the Gujarat coast were similar to *Rhodopseudomonas*, *Oligotropha*, *Nitrosospira*, *Rhizobium*, *Salinisphaera*, *Alcaligenes*, *Pelomonas*, *Paracoccus*, *Rhodobacter*, *Agrobacterium*, *Sinorhizobium*, *Ochrobactrum*, *Aurantimonas*, *Methylocapsa*, *Bradyrhizobium*, *Azospirillum*, *Nitrosospira*, *Sulfobacillus*, *Mycobacterium* sp. (Yousuf et al. 2012).

From Ganjam (Orissa), out of 355 paddy rhizospheric bacteria, five belonging to *Bacillus*, *Microbacterium*, *Methylophaga*, *Agromyces* and *Paenibacillus* sp. were potent PGPB which produced ACC deaminase (603.94 to 1350.02 nmol α -ketobutyrate/mg protein/h indole acetic acid (IAA) (10.54–37.65 $\mu\text{M}/\text{ml}$) and ammonia, and 2 isolates produced siderophores. The organisms enhanced growth of rice (*Oryza sativa* L.) variety Naveen (Bal et al. 2013).

From the coastal saline soils of Kumta (Karnataka), Lakshman et al. (2013) reported that the arbuscular mycorrhizal (AM) fungus *Glomus macrocarpum* enhanced plant survival, their PGPR functions with gypsum could reclaim coastal sand dune soil on saline-alkaline affected soils i.e., they could help in revegetation of such ecosystems.

11.11 Microbial Dynamics of Inland Saline Soils of India

The inland salinity severely affects microbial functionalities and the potent salt tolerant PGPRs would be helpful to maintain ecosystem functions, and soil and plant health to sustain productivity. However, each ecology has well established native microbial consortia which continuously adapt for own survival and in the process support ecosystem functioning. All over the world, different workers have reported osmotolerant PGPB which are effective to support plant growth. For example, from saline sodic soils of Rae Bareilly district (Uttar Pradesh), Damodaran et al. (2013) recorded rhizobacteria of $0.5\text{--}9.0 \times 10^5$ cfu/g root zone soil of 16 halophytes, out of which 31% tolerated 7.5% NaCl, 44% produced IAA and HCN, 25% produced siderophore, 56% produced NH_3 and 50% solubilized phosphate and the potent PGPRs were *B. pumilus* and *B. subtilis* that elicited significantly higher vigor index in tomato seedlings grown in pot culture experiments under saline sodic soils of pH 9.35 and EC 4.2. Upadhyay et al. (2009, 2012) reported bacterial population of

6.9×10^6 cfu/g soil in saline sodic soils of Varanasi, Mau, Ballia and Ghazipur (Uttar Pradesh) out of which about 33 % survived in $>8\%$ NaCl (w/v) and 19% showed PGP attributes (ACC deaminase, IAA, GA and siderophore production and phosphate solubilisation) at higher NaCl concentration. The ten potent salt tolerance PGPRs were identified as *B. aquimaris*, *B. pumilus*, *B. arsenicus*, *B. sporothermodurans*, *B. cereus*, *B. aquimaris* and *B. subtilis*, *Arthrobacter* sp., *Pseudomonas mediconae*. Proline played the role of osmotolerance of the organisms. (Upadhyay et al. 2009, 2012).

Similarly, the PGPR isolates from Gujarat viz., *Bacillus pumilus* and *Pseudomonas pseudoalcaligenes* could help to alleviate salt stress in the paddy (*Oryza sativa*) in salt-stressed plants resulting in 16% higher germination, 8% higher survival, 27% higher dry weight, and 31 % higher plant height and the PGPR inoculated rice plants also showed increased concentrations of N (26%), P (16%), K (31 %), and reduced concentrations of Na (71 %) and Ca (36%) under saline conditions (Jha and Subramanian 2013).

11.12 Microbial Dynamics of Salt Lake Ecosystems of India

Microbiology of the salt lakes is most poorly understood, and other than the Lonar and Sambhar lakes, none have been studied for microbial activities. From Sambhar Lake, moderate halophile *Halofarux volcani*, and several red extreme haloalkalophilic, anoxygenic, photosynthetic, sulfur oxidizing red bacteria viz., *Natrialba*, *Ectothiospira* etc. were reported by Upasani (2008). Besides, it was observed that, out of 74, 55 bacterial isolates of Sambhar Lake showed K-solubilizing and had various PGP activities.

The Lonar lake sediment recorded 86 halophilic bacteria namely *Halomonas*, *Alkalibacillus*, *Dietzia*, *Vagococcus*, *Exiguobacterium*, *Bacillus*, *Cellulosimicrobium*, *Thermoactinomyces*, *Alkalimonas*, *Penibacillus*, *Marinobacter*, *Roseinobacter*, *Rhodobacteriaceae* and *Rhizobium* sp. which have diverse PGP functions (Kaneekar et al. 2008). Deshmukh et al. (2011) isolated 3–15% salt tolerant *Planococcus maritimus*, *B. cohnii*, *B. subtilis*, *B. licheniformis*, *Alcanivorax* sp., *Oceanobacillus iheyensis* and *Haloalkaliphilic* sp. which have industrial potential like pigment, antibiotic, protease, amylase, lipase etc. producer and hydrocarbon degraders. Non-symbiotic nitrogen fixing bacteria viz., *Azospirillum lipoferum* which produced indole acetic acid, tolerate 4 % salt and alkaline pH 8, and enhance *Vigna* seed germination were also documented (Hingole and Pathak 2013).

11.13 Functional Diversity of Salt Tolerant Microbes

Bacteria colonize the rhizosphere and plant roots, stems, leaves and inside the plants and enhance plant growth by direct or indirect mechanisms referred to as PGPR. PGPR functions by synthesizing particular compounds for the plants, facilitating

the uptake of certain nutrients from the soil, and protecting the plants from diseases and pests etc. (Glick 1995, 2001; Whipps 2001; Glick and Pasternak 2003; Kumar et al. 2011). Under salt stress, different PGPB enhance germination rate, tolerance to drought, weight of shoots and roots, yield and plant growth (Kloepper et al. 2004; Kokalis-Burelle et al. 2006). The beneficial attributes of these organisms, such as, antagonism against bacterial and fungal pathogens of agricultural crops, nutrient cycling in the rhizosphere microcosm and root colonization are some of the challenges under stress (Zhou et al. 2008). Furthermore, it was well established that several rhizobia can tolerate pH 10.3–12, NaCl 3–28%, temperature up to 50 °C which can support plant growth under stress conditions (Tilak et al. 2005).

11.13.1 Toxin and Inhibitor Producing Osmotolerant PGPB

These groups of microbes are significant for control of pests and diseases, inhibition of other microbes and support plant growth. The *B. thuringiensis* (Bt) and *Pseudomonas* sp. (Ps) are the two most potent plant colonizing organisms which have polyvalent functions viz., PGP, biocontrol, stress endurance etc. (Whipps 2001; Barea et al. 2005; Botelho and Mendonca-Hagler 2006; Raddadi et al. 2009; Francis et al. 2010). A number of hydroxyl phenazine compounds with broad-spectrum antibiotic activity are metabolized by the *Pseudomonas*, *Streptomyces*, *Nocardia*, *Sorangium*, *Brevibacterium*, *Burkholderia* sp. etc. and AHL lactonase production by Bt which can support plant growth promotion and protection (Tambong and Hofte 2001; Raddadi et al. 2009).

11.13.1.1 PGP Functions of *Pseudomonas*

Many *Pseudomonas* strains of saline ecologies produce secondary metabolites which control of bacteria, fungi, algae etc. (Saikia et al. 2006; Whipps 2001). The most important principle for anti plant pathogens produced by *Pseudomonas* sp. is siderophore mediated competition for iron (Henri et al. 2008). Other antagonism potential of the pseudomonads are attributed to phenazines, pyocyanin, phenazine-1-carboxylic acid (PCA), phenazine-1-carboxamide (PCN) and a number of hydroxyl phenazines encompass a large family of heterocyclic nitrogen-containing brightly colored pigments pyoverdines, HCN, organic acids (salicylic acid), pathogenicity related (PR) enzymes like peroxidase, antibiotics like bacteriocins, 2, 4-diacetylphloro glucinol (DAPG), pyrrolnitrin complex macrocyclic lactone etc (Saikia et al. 2006; Srivastav and Shalini 2008). Phenazine derivatives not only kill fungi but the phenazine derivative pyocyanin, produced by certain *P. aeruginosa* strains, also kill of animal cells and tissues (Mahajan et al. 1999). Other mechanism are: the systemic acquired resistance (SAR), is associated with increased level of salicylic acid, jasmonic acids, ethylene etc. and activation of pathogenesis-related (PR) proteins (Gaffney et al. 1993; van Loon 1997; van Wees et al. 1997) and induced systemic resistance (ISR) where the plant defense mechanism induced by non-pathogenic biocontrol bacteria viz., rhizobacteria (van Loon 1997; Pieterse et al.

2001) activated by lipopolysaccharides, siderophores or the flagella components (Maurhofer et al. 1994; Leeman et al. 1995).

11.13.1.2 PGP Functions of *Bacillus thuringiensis* (Bt)

B. thuringiensis a ubiquitous bacteria present in agricultural and fallow, forest, garden soil etc., water, air, phyllosphere, diseased insects, stored products all over the world accounting for $0\text{--}128 \times 10^4$ cfu/g substrate and its population in soil is generally lower than the phyllosphere. In rice field soils, Bt density ranged within about $5\text{--}66 \times 10^4$ cfu/g soil and its index varied within 0.002–0.006 (Das and Dangar 2008; Dangar et al. 2010). Recently, different researchers reported variable osmotic stress (4–18 % NaCl) tolerance of Bt (Kaur and Singh 2000; Das and Dangar 2008; Dangar et al. 2010). The Bt isolates of each soil differed for salt tolerance i.e., some of them tolerated 2–5 % NaCl like normal mesophilic microbes, 6–9 % salt, whereas, others tolerated 12 % NaCl which gives an direct idea that halotolerance of Bt had no relation with habitat (Das and Dangar 2008). Salinity is the major concern in the coastal agricultural zones in terms of its negative effect on the sustainability of beneficial microorganisms associated with the rhizosphere. Different Bt strains (83 known serotypes) with different types of toxins (300 alleles of 51 different types) like δ -endotoxins (also known as crystal (Cry) toxins, proteases, lipases, cytolysin (Cyt 1–17), vegetative induced proteins (VIP), AHL lactonase, siderophore, bacteriocins, etc. which control different pests or diseases (Zeigler 1999, Raddadi et al. 2009) were isolated from diverse ecologies in India and found to be effective PGP function for rice growth and control of the pests (Das and Dangar 2008; Das et al. 2008; Dangar et al. 2010).

11.13.2 Nitrogen Fixing PGPB

Nitrogen-fixing (diazotrophic) bacteria fix atmospheric nitrogen (N_2) through the enzyme nitrogenase, a two component metallo enzyme composed of dinitrogenase reductase, a dimer of two identical subunits that contains the binding sites for Mg-ATP and hydrolysis, and supplies the reducing power to the dinitrogenase that contains a metal cofactor (Subba Rao 2007; Chavada et al. 2010; Alexander 2011; Barua et al. 2011). Salinity decreases mineralization and immobilization of nitrogen, nitrification and ammonification (Subba Rao 2007; Alexander 2011). It has been observed that as the saline habitats are nitrogen poor, therefore N input is very important in this environment (Subba Rao 2007; Alexander 2011; de-Bashan et al. 2012). Ammonia or nitrates in assimilatory forms in saline soil are important for salt tolerant bacteria which take them as osmolytes. Reasonably, prevalence of the nitrogen-fixing bacteria (*Azotobacter* sp.) in the sediments of Pichavaram mangrove habitat than in the marine backwaters and estuarine systems would be effective for nitrogen replenishment (Lakshmanaperumalsamy 1987). Two halotolerant, nitrogen fixing *Rhizobium* sp. have also been isolated from root nodules of *Derris*

scandens and *Sesbania* sp. growing in the mangrove swamps of the Sundarbans (Sengupta and Chaudhuri 1990). Presence of nitrogen fixing bacteria and cyanobacteria in Pichavaram mangrove is very essential for maintenance of the fragile ecology (Lakshmanaperumalsamy 1987; Ravikumar 1995).

11.13.3 Phosphate Solubilizing PGPB

After nitrogen, phosphorous is the second major nutrient for plant growth as it is an integral constituent of different biochemicals like nucleic acids, phospholipids and phosphor-proteins (Kim et al. 1998; Rodriguez and Fraga 1999). The concentration of soluble P in soil is usually very low (1 µg/ml or less), but P in soil is present in two main insoluble forms: mineral forms such as apatite and hydroxyapatite, and oxyapatite and organic forms including inositol-phosphate, phosphomonoesters, phosphodiester and phosphotriesters (Loganathan and Nair 2003; Rodriguez et al. 2006). Salinity decreases P accumulation in plant and developed P-deficiency symptoms which is the result of ionic strength effects that reduce the activity of phosphate (Subba Rao 2007; Alexander 2011; de-Bashan et al. 2012). The cell might take up several P forms but the major part is adsorbed in monobasic and dibasic forms. The major mechanism used by phosphate solubilizing bacteria (PSB) for solubilization of inorganic P is based on the synthesis of low molecular weight organic acids such as gluconic, citric acid etc. (Rodriguez et al. 2006). These organic acids bind phosphate with their hydroxyl and carboxyl groups thereby chelating cations and also induced soil acidification, both resulting in the release of soluble phosphate (Kim et al. 1998; Rodriguez et al. 2006). Besides, exopolysaccharides synthesized by PSB participate indirectly in the solubilization of tricalcium phosphates by binding free P in the medium, affecting the homeostasis of P solubilization (Yi et al. 2008). The mineralization of organic P occurs through the synthesis of phosphatases, including phosphomonoesterase, phosphodiesterase and phosphotriesterase, catalyzing the hydrolysis of phosphoric esters. In addition, P solubilization and mineralization can co-exist in the same bacterial strain.

11.13.4 ACC (1-aminocyclopropane,1-carboxylate) Deaminase Producing PGPB

Salinity stress enhances ethylene production in plants which is a stress hormone (Glick 1995; Glick et al. 1998; Chakraborty et al. 2011). As it is a senescence-inducing hormone, their higher concentrations have inhibitory effects on root growth and plant development. Chakraborty et al. (2011), by inoculation with ACC deaminase positive bacteria, reported that ACC deaminase bacteria confer salt tolerance of plants by lowering the synthesis of salt induced stress ethylene and could promote canola growth in saline environments and maize growth under salt stress (Glick 1995; Glick et al. 1998; Chakraborty et al. 2011; Bal et al. 2012, 2013).

Table 11.7 Different osmolytes produced by the osmotolerant microbes

Compatible solutes	Organism
Ectoine	<i>Halomonas elongata</i> , <i>H. boliviensis</i> , <i>Ectothiorhodospira halochloris</i> , <i>Brevibacterium epidermis</i> , <i>Chromohalobacter israelensis</i> , <i>C. salexigens</i>
Hydroxy ectoine	<i>H. elongata</i> , <i>Nocardiopsis halophila</i> , aerobic heterotrophic bacteria
Betaine	<i>Actinoploypora</i> sp., <i>Halorhodospira halochloris</i> , <i>Thioalkalivibrio versutus</i>
Proline	<i>Streptomyces</i> , halophilic/halotolerant <i>Bacillus</i> strains
Trehalose	<i>Pyrobaculum aerophilum</i> , <i>Thermoplasma acidophilum</i> , <i>Actinopolypora halophila</i> , <i>Rubrobacter xylanophilus</i>
Diglycerol phosphate	<i>Archaeoglobus fulgidus</i>
Sucrose	<i>Anabaena</i> (blue green algae), <i>Nitrosomonas europaea</i> and proteobacteria
Mannosylglyceramide	<i>Rhodothermus marinus</i>
Mannitol	<i>Pseudomonas putida</i>
Mannosylglycerate	<i>Methanothermus fervidus</i> , <i>Pyrococcus furiosus</i> , <i>Rhodothermus marinus</i> , <i>Thermus thermophilus</i> , <i>Pyrococcus furiosus</i> , <i>Thermococcus</i> sp.

From rice rhizosphere, efficient PGPRs viz., *Bacillus*, *Microbacterium*, *Methylophaga*, *Agromyces* and *Paenibacillus* sp. capable to produce both ACC deaminase (603.94–1350.02 nmol α -ketobutyrate/mg/h) and IAA (10.54 to 37.65 μ M/l) enhanced root elongation inoculated by rice (*cv.* Naveen) seed treatment. Some of them also possessed ammonia and siderophore production properties. Such PGPRs may be exploited under field conditions for a sustainable crop management (Bal et al. 2012, 2013).

11.14 Adaptation of Bacteria in Response to Salinity

Osmotic stress differentially effects metabolism of the microbes and surprisingly, hyper-osmoticum of NaCl and sea salt (natural mixture of salts) have differential effects on physiology of different microbes. Although not universal, sea salt exerts lesser stress on the microbes than NaCl. Microorganisms adopt the principle mechanisms like regulation of Na⁺ ions, compatible solute (osmolyte) and anti-oxidative enzymes (osmozymes) to endure stress (Khan et al. 2003; Moat et al. 2002; Das and Dangar 2008). To lower the chemical potential of cell water for osmotic adaptation, in one mechanism i.e., the salt-in-cytoplasm mechanism, the microbes viz. the sulfate reducers, Archaea, fermenting bacteria, acetogenic anaerobes (e.g., *Haloanaerobium*, *Halobacteroides*, *Sporohalobacter*, *Acetohalobium* sp. etc.) raise salt concentration in the cytoplasm and the interior proteins adapt to high salt concentration (Moat et al. 2002). Whereas, in the other mechanism i.e., the organic-osmolyte mechanism, the organisms (Bacteria, Eukarya and some methanogens) maintain almost NaCl-free cytoplasm i.e., cell's interior remains unchanged and the cell water is reduced by uncharged, highly water-soluble organic solutes (ectoine, choline, betaine, proline, glutamic acid, other amino acids, regulators, elevate

potassium ions, sugars, alcohols, amino acids, betaines, ectoines or their derivatives) (Table 11.7) accumulated either by *de novo* synthesis or by uptake from the surrounding environment (Moat et al. 2002). Compatible solutes maintain osmotic equilibrium across the cell membrane and stabilize proteins and even whole cells to protect against heat, desiccation, freezing and thawing, and denaturants such as urea and salt (Moat et al. 2002). However, microbes also maintain the stress affected membrane integrity through the anti-oxidative enzymes like catalase, peroxidase, polyphenol oxidase, ascorbate peroxidase and ascorbic acid oxidase etc. The salt tolerant (10–13 % NaCl and sea salt) bacteria of Bhitarkanika endure stress by metabolic regulation through catalase, peroxidase, polyphenol oxidase, ascorbate peroxidase and ascorbic acid oxidase which tolerated 600–1000 ppm K_2CrO_4 but 10–20 ppm $CdNO_3$ stress also (Mishra et al. 2009). It was observed that the osmolytes (mg/g dr. wt.) like the amino acids (0.38–99.45) and proline (0.38–0.80); and the anti-oxidative enzymes (units (U)/mg protein/min) viz. the catalase (0.17–5.59) and superoxide dismutase (0.35–74.46) effected intrinsic osmotic stress tolerance of the Bt (Das and Dangar 2008). Microbes like *E. coli*, *Arthrobacter*, *Bacillus* sp. etc. have evolved genes, which are regulated osmotically at the transcriptional level for uptake/synthesis of specific osmolytes (Moat et al. 2002).

11.15 Osmosensor and Osmoregulation by Bacteria

Osmosensor macromolecules undergo conformational transitions “off” and “on” in response to changes in extracellular water activity or resulting changes in cell structure. According to RoeBler and Muller (2001), bacterial responses to osmolarity shifts are on the genetic and enzymatic level known as two component regulatory systems which consist of a sensor protein, that detects the signal, and a regulator protein that binds DNA and controls gene expression. The two component regulatory systems that respond to the osmotic changes are the EnvZ/OmpR and the KdpD/KdpE (Quin et al. 2000). The EnvZ/OmpR has been found in the Eubacteria while the KdpD/KdpE occurred in Archaea. The EnvZ is a transmembrane histidine kinase that monitors osmolarity changes on both sides of the cytoplasmic membrane through the OmpR (Bartlett and Roberts 2000). It functions as a dimer with a part in the cytoplasm and a part extending outside the membrane. It has three separate enzymatic activities. The KdpD, is auto-phosphorylated under a decrease in turgor pressure condition. Subsequently, the phosphoryl group is transferred to the response regulator, KdpE, which then acts as a transcriptional activator for the KdpABC operon encoding a primary ABC-type K^+ transporter. This allows the cell to counteract the stress by increasing the internal osmolarity through the accumulation of K^+ via the KdpABC ATPase (Moat et al. 2002).

11.16 Applications of Osmotolerant Microbes

Microbial diversity is the key to human survival and economic well being and provides a huge reservoir of resources which we can utilize for our benefit. Marine mangrove fungi have commercial sources of xylanolytic enzymes used in industries, such as in paper manufacturing, animal feed, bread making, juice and wine industries and xylitol production (Polizeli et al. 2005). Marine algae are rich sources of food, feed, medicines and energy. Marine algae are the only sources for industrially important phycocolloids like agar, carrageenan and alginate (Sahoo and Dhal 2009), blood anti-coagulant, anti-tumor, anti-mutagenic, hypoglycemic, antiviral, anti-complementary, hypolipidemic, immunomodulating and anti-inflammatory activities. Actinobacteria of mangroves are source of anti-infection and anti-tumor compounds, neurodegenerative disease and diabetes treatment (Hong et al. 2009). Many bacterial strains contain genetic determinants of resistance to heavy metals such as cadmium, silver, mercury, arsenic, bismuth, chromium, nickel and lead etc. and useful to detoxify such pollutants (Sahoo and Dhal 2009; Mishra et al. 2011).

Mangroves are rich sources of antibiotic, antitumor, enzyme i.e., cellulase, glutaminase etc. producing actinomycetes. From Veller estuary, the actinobacteria viz., *Streptomyces griseobrunneus*, *S. galbus*, *S. alboniger*, *S. vastus*, *S. violaceus*, *S. moderatus* and *S. aureofaciens*; from Pichavaram *S. albidoflavus* that produce antitumor compounds and from the West coast mangroves *Actinopolyspora* sp. that showed wide antimicrobial activity against *Staphylococcus aureus*, *S. epidermis*, *B. subtilis*, *Aspergillus niger*, *A. fumigates*, *A. flavus*, *F. oxysporum*, *Penicillium* sp., *Trichoderma* sp. etc. were recorded. However, they had no effect on the Gram-negative bacteria i.e., *E. coli*, *P. aeruginosa*, *S. marcescens*, *E. aerogenes*, and fungi like *C. albicans* and *Cryptococcus* sp. (Sahoo and Dhal 2009; Mishra et al 2012a, b). Thus, microbes have immense utility in all spheres of human civilization.

11.16.1 In Fermented Food Industry

Halotolerant microorganisms catalyze the fermentation in presence of salt; thereby produce various compounds that give the characteristic taste, flavor and aroma to the resulting products (Margesin and Schinner 2001). In the production of pickles (fermented cucumbers) brine strength is increased gradually from 5–15.95% (w/v) NaCl. Fermentation of sauerkraut (pickled cabbage) occurs in the presence of 2.25%–2.5% salt. *Lactobacillus plantarum* is the most essential species in both processes. *Halobacterium salinarum*, *Halococcus* sp., *Bacillus* sp., *Pseudomonas* sp. and coryneform bacteria are used in the production of an Asian (Thai) fish sauce in which fish is fermented in concentrated brine (Thongthai and Suntinanalert 1991).

Salt tolerant microbes viz., *Tetragenococcus* strains are used for soy sauce fermentation in about 19% NaCl solution up to 9 months and the microbe grow up to 10^8 cfu/ml in soy sauce mash with about 3 M NaCl. Microbial enzymes from the mangrove environment have wide applications. About 71 % bacterial strains (viz.,

Halococcus sp.) produce L-asparaginase having higher anti-leukemic activity in humans than that of non-mangrove sources (Mishra et al. 2012b). Arylsulfatase which metabolize sulphuric acid esters is produced predominantly by *Bacillus*, followed by *Vibrio* and the activity ranges from 21.61–32.61 g phenolphthalein/g sediment, in the mangrove followed by the backwater and estuary, especially during summer and premonsoon periods (Sahoo and Dhal 2009). Besides phosphatase activity, capable of solubilizing phosphate (Mishra et al. 2012b), the mangrove sediments exhibit higher activity of L-glutaminase than sediments from other biotopes and clayey sediments show higher enzyme activity than silty ones (Sahoo and Dhal 2009).

11.16.2 Compatible Solute Production

The moderate halophiles and osmotolerant microbes produce and accumulate high concentrations of compatible solutes which are useful for the biotechnological production of stress protectants against high salinity, thermal denaturation, desiccation, freezing and stabilizers of enzymes, nucleic acids, membranes and whole cells, enzyme technology (biosensor technologies, PCR etc.), pharmaceuticals and cosmetics etc. The glycine betaine, trehalose, glycerol, proline, ectoines and sugars, hydroxyectoine protect lactate dehydrogenase against freeze-thaw treatment and heat stress whereas ectoine is effective freeze-stabilizing agent for phosphofructokinase (Ventosa et al. 1998). A novel biotechnological process called ‘bacterial milking’ has been established for the production of betaine, ectoines and hydroxyectoines compounds by the extremely halotolerant *Halomonas elongata* (Margesin and Schinner 2001). Advances in fermentation technology and genetic engineering of moderate halophiles will allow production of compatible solutes, cloning of these for salt and drought tolerance to agricultural crops, such as wheat, rice and barley, would enhance growth in more saline soils.

11.16.3 In Polymer Production

Bacterial polysaccharides enhance oil recovery as surfactant and bioemulsifying properties. Since the conditions in oil deposits are often saline, the salt-resistant surfactants may be advantageous. More than 200 bacterial strains are capable of producing extracellular polysaccharides from oil wells and oil well-associated environments. Bacteriorhodopsin, the retinal protein proton pump of *Halobacterium* is being explored in photochemical processes.

11.16.4 In Bioremediation, Biosurfactant and Bioplastic Production

The salt tolerant microbes have extensive use in bioremediation of iron and heavy metal, pesticides, detergents, agrochemicals, from industrial, agricultural, domestic wastes; food industry, oil field, leather industry etc. as the osmotolerant microbes can overcome the stress imparted by the polluted effluents. A large number of microbes viz., *E. coli*, *Pseudomonas*, *Bacillus*, *Arthrobacter*, *Sphingomonas*, *Enterobacter* sp. etc. are extensively used for bioremediation.

Biosurfactants enhance the remediation of oil contaminated soil and water. Biosurfactant producing halophilic/halotolerant microorganisms may thus play a significant role in the accelerated remediation of oil-polluted saline environments (Banat et al. 2000). One of the most effective biosurfactant is lichenisin, a cyclic lipopeptide, produced by *Bacillus licheniformis* JF-2. Polyhydroxyalkanoates (PHA), the biodegradable plastics could replace oil derived thermoplastics in some fields. *Haloferax mediterranei* accumulates large amounts of poly β -hydroxy butyric acid (PHB).

11.16.5 Use in Agriculture

Salinity is a major problem in world agriculture as the saline soils are nutrition poor. Salt tolerant genotypes of most crops are not readily available for sustainable production. Microbes are the most efficient natural but poorly exploited for crop production. The salt tolerant PGPRs viz., *Pseudomonas*, *Bacillus*, *Azotobacter*, *Azospirillum*, *Azomonas* sp., besides various P, N, S, micronutrient metabolizing microbes, biocontrol agents etc. have the promise to maintain ecological functioning under stress conditions (Rangarajan et al. 2003; Sahoo and Dhal 2009; Samuel and Muthukkaruppan 2011). For example, rice (*Oryza sativa*) production depends highly on N and P fertilizers which are the limiting factors for production. Therefore, the free-living nitrogen-fixing bacteria viz., *Azotobacter* and *Azospirillum* sp., P metabolizing microbes viz., *Pseudomonas* sp., bioagents with different PGP functions viz., metabolism of plant hormones, macro/micro nutrients, toxins, inhibitors etc. would be the best alternative of chemical fertilizers (Mishra et al. 2012a, b; Sahoo et al. 2013a, b, c).

11.17 Conclusion

Salinity is one of the most critical, ill understood but integral and most abundant ecology on the Earth. However, ecology has its own well adapted resident microflora. The microbes of these ecologies also perform all functions of life for survival of their own and associated biological entities. Stress tolerant organisms have evolved

the capacity to function under so called unusual conditions. Understanding and exploitation of the novel characters of these microbes would provide ample scope to improve and sustain agricultural and industrial productivity, monitor and regulate anthropogenic detrimental activities that affect biological and environmental health. Therefore, the osmotolerant microbes are the ignored natural resources yet to be exploited for human benefit. An account of the stress ecologies, dynamics, diversity and functionalities, and present status of their utilization would help to plan for all round development in future by exploiting these golden resources of great promise for development of agriculture, industry, environment etc.

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Chapter 12

Culture Independent Diversity Analysis of Soil Microbial Community and their Significance

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Abstract Soil is considered as one of the richest source of microbes on the earth. Understanding microbial diversity of soil is a daunting and challenging task. The culture-independent techniques have revealed that the soil microbial diversity is much larger than was understood through culture-dependent techniques. Various DNA- and RNA-based techniques have revolutionized our understanding of soil microbial diversity. This chapter deals with the major culture-independent methods used for the study of soil microbial diversity, their advantages and limitations and advent of metagenomics in microbial diversity studies. Significance of culture-independent techniques in conservation of microbes has also been discussed.

12.1 Introduction

The microbial diversity is described as the number of different species and their relative abundance in a given community in a given habitat. The soil is considered as one of the major reservoirs of microbial diversity on our planet (Swift et al. 1998) with an estimated bacterial diversity of 10^4 to 10^6 taxa per 1 g of soil (Torsvik et al. 1990; Gans et al. 2005). Torsvik and Overeas (2002) found that 1 g of soil has 4000 different species of microbes and the bacterial population in top layers of soil can be more than 10^9 individual cells per gram soil. Microorganisms in soil are critical to the maintenance of soil function because of their involvement in key processes like soil formation, decomposition of organic matter, toxin removal and the cycling of carbon, nitrogen, phosphorus, and sulphur (van Elsas and Trevors 1997). Besides soil functions, microbes especially bacteria, are involved in every sphere of human life ranging from industry, medical, food, bioremediation, production of energy, mining and so on. Perhaps, one of the most significant gifts of microbes to human society is antibiotics. Various research works have shown that microbes play an important role in the ecosystem functioning and hence an understanding of the microbial interaction with its environment is needed to help us to improve our bioresources. For example, plant health improves by use of plant growth promoting

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rhizobacteria (PGPRs) and livestock by use of probiotics etc. Now-a-days, microbes are found to have the potential of producing non-conventional source of energy like biogas and hence they can be considered as environment friendly energy sources too. However, in order to harness and utilize microbial resources for our improved lifestyle, we must first have knowledge of the microbial diversity surrounding us.

From the times of Leeuwenhoek until recently, the conventional approach of characterization of soil microbial diversity usually involved application of culture-based techniques using a wide variety of culture media and prolonged incubation periods for isolation of diverse microbial groups. Less than 1% of total bacterial population could be cultured by using these techniques (Vartoukian et al. 2010). Ability to isolate a small fraction of bacterial species with specialized growth requirements is major handicap of this approach. This concept, therefore, cannot detect the dominant uncultivable soil microbial communities, although significantly higher number of such organisms has been recorded (Pace 1997) than the culturable ones. Recently, Bhattacharyya et al. (2013) emphasized the need of culture-independent metagenomic approach for the exploration of unexplored microbial gene pool. The microbial world explored so far using the culture-dependent methods seems to be only a meager part of what lies beneath. This view is supported by “Great Plate Count Anomaly” of Staley and Konopka (1985) who demonstrated that bacteria though easy to cultivate in laboratory, represent only a small fraction of the microbes present in the environment. Soil microbial communities are often difficult to be fully characterized mainly because of their immense phenotypic and genotypic diversity, heterogeneity, and crypticity (Garbeva et al. 2004). Hence, the conclusion that estimation of soil microbes by culture-based methods is insufficient can be accepted. This fact is further supported by the findings of Torsvik and Overeas (2002) that only 0.1–1% of bacteria is readily culturable on common media under normal conditions.

The soil microbial diversity is considered as one of the richest source of microbes which can be harnessed for human welfare. These microbes can prove to be a rich source of various biochemicals (e.g., antibiotics, toxins etc.), enzymes etc. but for obtaining these resources a thorough exploration of all the microbes present in the soil is required. The isolation and characterization of microorganisms belonging to widespread but previously uncultivated groups of organisms can provide insights into the roles and functions of these organisms in their natural settings and can assist in the formulation of hypotheses about metabolic interactions among microorganisms in the natural environment (Zinder and Salyers 2001). The exploration of all the unknown microbes will enable microbiologists to compare microbial communities within and across different ecosystems and utilize this knowledge for various purposes like biotechnological applications, ecosystem restoration etc.

The introduction of 16S rRNA and direct DNA-based culture-independent methods to assess the total microbial diversity has overcome the limitations of culture-dependent studies and now microbial diversity studies are possible in different environments like soil, air, water, cheese, human gut etc. otherwise considered difficult (Dethlefsen et al. 2008; Jany and Barbier 2008; Tringe et al. 2008; Gugliandolo et al. 2010; Berlemont et al. 2011). The discovery of 16S rRNA gene

applications in the microbial studies revolutionized this field and consequently has enabled workers to explore the depths of microbial diversity. The highly conserved nature of 16S rRNA among different bacterial species, universal distribution and immune to horizontal gene transfer (Daubin et al. 2003) makes it suitable for phylogenetic studies.

Over the last 20 years, amplification and sequencing of the small subunit ribosomal RNA (SSU rRNA) or 16S rRNA gene has been the primary approach to assess the abundance and taxonomic identity of bacteria in the environment. Based on its universal presence and relatively uniform rate of evolution, SSU rRNA enabled the discovery and classification of a vast diversity of uncultivated microorganisms spanning all phylogenetic levels (DeLong and Pace 2001; Tringe and Hugenholtz 2008; Pace 2009). The sequencing surveys of amplified regions of SSU rRNA genes have revealed that microbial diversity is much greater than the 5000 microbial species described using phenotypic features in Bergey's taxonomic outline (Guerrero 2001), and that microbial communities are far more complex than initially thought. For example, a study by Huber et al. (2007) using short hypervariable regions of 16S rRNA gene demonstrated that there are over 40,000 different kinds of bacteria and archaea in a few ml of hydrothermal vent fluid.

Though 16S rRNA is widely accepted for assessment of microbial diversity, gradually it is observed that it shows some variation among the strains of the same species (Acinas et al. 2004) and a single rRNA gene may have a copy number of 1–15 or more (Klappenbach 2001). This led to the estimation of almost 2.5 fold greater than the actual number of bacterial cells present in the sample (Acinas et al. 2005). Both multiplicity and variability represented problems for the assessment of bacterial diversity and community structure as currently bacterial communities are analyzed by construction of similarity clusters or operational taxonomic units (OTUs) of 16S rRNA gene PCR amplicons. The variability of 16S rRNA skews the abundance estimates of a taxon and the multiplicity affects diversity estimates, thereby, making the relative abundance in complex bacterial population unreliable (Farrelly et al. 1995). Until recently, it was difficult to draw conclusions about the extent of the biases caused by the use of 16S rRNA analyses. The limited amount of sequenced bacterial genomes and the low resolution of the 16S rRNA gene for differentiation of closely related species, often make the diversity analysis results biased. Further, Větrovský and Baldrian (2013) showed that interspecific or intergeneric similarity in 16S rRNA gene sequences can also significantly affect OTU construction resulting into falling of few species or even genomes to different OTUs, if the standard level of 16S rRNA similarity of 97% is used. Likewise, species of several genera will not be separated at the same similarity level because the 16S rRNA differences were lower than 97%. This, however, depends on the part of the 16S rRNA gene used for OTU construction. It was also observed that the various experimental and computational steps developed for diversity characterization using 16S rRNA gene's hypervariable regions are prone to errors (Hong et al. 2009; Huse et al. 2010; Haas et al. 2011). Amplification of different hypervariable rRNA gene regions can lead to inconsistent taxonomic coverage and incongruence between data sets. In addition, short read amplicon sequencing requires specific considerations

for the methodology, data analysis and interpretation of microbial diversity (Quince et al. 2011; Schloss et al. 2011; Gihring et al. 2012; Werner et al. 2012).

Along with 16S rRNA gene, difference in the guanine plus cytosine (G+C) content of DNA can also be used as a helping tool to study soil bacterial diversity (Nüsslein and Tiedje 1999). As it is known that taxonomically related microbes differ in their G+C content by 3–5% only (Tiedje et al. 1999; Bing-Ru et al. 2006), hence, this method can be considered as a preliminary tool for diversity analysis. An advantage of this approach is that there is no PCR bias and hence some of the less dominant microorganisms in the community which couldn't be detected by PCR, can also be detected and analyzed.

Thus, the employment of 16S rRNA gene sequences and G+C (mol%) made the basis of culture independent studies of microbial diversity. The pioneering work of Woese (1987) emphasizing that direct analysis of 5S and 16S rRNA gene sequences could be used to describe diversity of microorganisms in an environmental sample without culturing them, laid the principle of culture independent studies (CIS). The next technical breakthrough came in the form of PCR technology and the design of primers which could be used to amplify almost the entire gene (Giovannoni et al. 1990). This accelerated the discovery of diverse taxa as these techniques made it easier for the microbiologists to study habitats across the earth (Giovannoni et al. 1990; Schmidt et al. 1991; Barns et al. 1999). These techniques further established the superiority of using 16S rRNA genes over conventional microscopy and fluorescent antibody studies for diversity analysis as there was no need of culturing of organisms and phylogenetic information could also be obtained. Hence, CIS can be considered as the new tool for studying microbial diversity across various habitats.

The objective of this review is to develop a comprehensive account of different methods available for microbial diversity analysis with special emphasis on the role of culture- independent methods and metagenomics in culture-independent studies. The interaction of culture independent methods or metagenomics for the estimation of microbial diversity and its conservation has also been looked into.

12.2 Culture-Dependent Methods

Different culture-dependent methods are used to study microbial diversity. These methods are classified as direct, physiological and biochemical methods. The direct method that includes plate counts, a classical but extensively used method, is inexpensive and provides direct information on the active component of the population (Tabacchioni et al. 2000). This method, however, faces limitations because it requires specific conditions like temperature, pH and light for the growth of the microbes. Further, only 0.1–1% of soil bacteria can be cultured under standard laboratory conditions using this technique (Torsvik et al. 1998).

The physiological means of analyzing diversity includes community level physiological profiles (CLPP) and sole carbon source utilization (SCSU) patterns. These profiles reflect how the microbial communities could potentially utilize a range of

carbon substrates. Differences in utilization patterns are interpreted as differences in the major active members of the microbial community. Although, the technique has become popular, yet there are certain limitations that make the analysis and interpretation of data so obtained complicated (Broughton and Gross 2000). For example the BIOLOG system used in this technique can assess the metabolic diversity of culturable bacteria only and hence, soil fungi and slow-growing bacteria are left untouched. The BIOLOG sole C-source test plates contain high concentrations of carbon sources (Campbell et al. 1997) and TTC (triphenyltetrazolium chloride) which are buffered at nearly neutral pH, thereby presenting a different environment for those microorganisms which are well adapted to acidic or alkaline soils. Thus, these bottlenecks limited the scope of this technique for determining soil microbial community structure in different habitats.

The biochemical methods viz. fatty acid methyl ester (FAME) and phospholipid fatty acid (PLFA) analyses are based on the presence of signature fatty acids in different microbial groups. FAME provides information about the microbial community composition based on groupings of fatty acids (Broughton and Gross 2000). Fatty acids make up a relatively constant proportion of the cell biomass, and signature fatty acids can differentiate major taxonomic groups within a community (Kirk et al. 2004). Therefore, a change in the fatty acid profile would indicate a change in the microbial biomass and community structure. The phospholipid fatty acid (PLFA) analyses technique is used to understand the strategies employed by microorganisms to adapt to changed environmental conditions under wide ranges of soil types, management practices, climatic conditions, and different perturbations (Zelles 1999). These bio-chemical methods being culture-dependent have limitations like they need specific growth conditions and can't be employed for culture of many fungi (van Elsas et al. 2000; Bing-Ru et al. 2006). Further these techniques are time consuming and require large number of samples.

These methods, therefore, have certain limitations and cannot be considered as suitable for diversity analysis of soil microbes as it is evident now that less than 1 % of microbes only are culturable. The advent of culture-independent methods thus, is a new approach for revealing the vast world of microbial diversity.

12.3 Culture-Independent Methods

The culture independent studies revolve around nucleic-acid and include analyses of whole genome or selected genes like 16S rRNA. Different culture-independent techniques have been developed after Pace et al. (1985) who proposed direct cloning of environmental DNA. These techniques can be used both for partial and whole community analysis. For the partial community analysis, PCR-based methods are used where the total DNA/RNA extracted from the environmental sample is used as a template for the characterization of the microorganisms. The whole community analysis involves non-PCR based techniques like hybridization, G+C content estimation, whole genome sequencing etc. The whole genome study techniques are

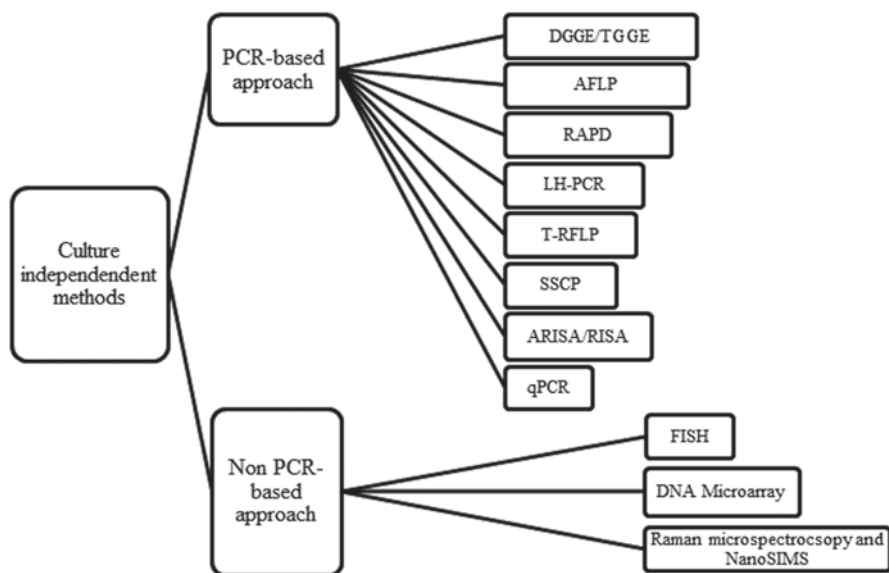


Fig. 12.1 Different culture independent methods to study microbial diversity

better than the PCR-based techniques as they provide a more comprehensive view of the genetic diversity by analyzing all the genetic information present in total DNA extracted from the sample. Most of the culture-independent methods used for microbial community diversity studies include sampling, DNA extraction, amplification of gene fragments from environmental samples, distinguishing different fragments, analysis and interpretation of experimental results and summary of microbial communities. Some of the frequently used culture-independent techniques in studies of soil microbial communities alongwith their advantages and limitations have been presented below (Fig. 12.1; Table 12.1).

12.3.1 *Random Amplified Polymorphic DNA (RAPD)*

Randomly amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) is a simple and rapid method used for analyzing genetic diversity and genetic relationships (Welsh and McClelland 1990; Williams et al. 1990). This technique utilizes PCR amplicons with very short primers (usually 10 bp) annealing at multiple locations on the microbial genome and produces a range of amplicons that are characteristic of the template. It generates PCR amplicons of various lengths in a single reaction that are separated on agarose or polyacrylamide gel depending on the genetic complexity of the microbial communities. It is one of the most commonly-used molecular techniques used for developing DNA markers. It is also less time consuming and cheaper than other molecular finger-printing methods and is suitable

Table 12.1 Different methods used for culture-independent microbial studies

Methods	Applications	Advantages	Disadvantages	Reference
RAPD	Determination of organismal genetic diversity at various taxonomic levels	Cost effective, high speed and ease of use, suitable for unknown genomes	Low reproducibility, sensitive to reaction conditions	Welsh and McClelland (1990), William et al. (1990)
AFLP	Identification of genetic variation in strains or closely related species	Simultaneous screening of different DNA regions distributed randomly throughout the genome.	Difficulty in identifying homologous markers	Vos et al. (1995)
DGGE/ TGGE	Study of microbial genetic diversity	Sensitive to variation in DNA sequences, bands can be excised, cloned and sequenced for identification	Time consuming, multiple bands for a single species can be generated due to micro-heterogeneity, can be used only for short fragments	Myers (1987) Yoshino et al. (1991)
SSCP	Study of microbial diversity	Screening of potential variations in sequences, helps to identify new mutations, - Enables analyses of a wide array of microbes	Short fragments lack of reproducibility several factors like mutation and size of fragments can affect the sensitivity of the method	Orita et al. (1989) Ravnik-Glavac et al. (1994)
T-RFLP	Identification of different species in a given community (biodiversity studies)	Highly reproducible, convenient way to store data and compare between different samples	Artifacts might appear as false peaks, distinct sequences sharing a restriction site will result in one peak, unable to retrieve sequences	Liu et al. (1997)
q-PCR	Measurement of abundance and expression of taxonomic and functional gene markers	Quick, accurate and highly sensitive method for sequence quantification that can also be used to quantify microbial groups, relatively cheap and easy to implement, specific amplification can be confirmed by melting curve analysis	Can only be used for targeting of known sequences, DNA impurities and artifacts may create false-positives or inhibit amplification	Bustin et al. (2005); Smith and Osborn (2009)

Table 12.1 (continued)

Methods	Applications	Advantages	Disadvantages	Reference
RISA	Comparison of microbial diversity in soil.	High resolution when detecting microbial diversity, quick and sensitive	More than one peak could be generated for a single organisms, similar spacer length in unrelated organisms may lead to underestimations of community diversity Limited by the bacterial species known in public databases	Fisher and Triplett (1999)
LH-PCR	Analysis of soil microbial communities	Results are reproducible easy and rapid efficient and reliable	Limited by the bacterial species known in public databases, not enough information is available for fragment length on databases to compare LH-PCR lengths with environmental microorganisms	Mills et al. (2007)
FISH	Enables <i>in situ</i> identification and enumeration of individual microbial cells	Allows detection and spatial distribution of more than one samples at the same time	Autofluorescence of microorganisms, accuracy and reliability is highly dependent on specificity of probe(s)	Amann et al. (1995)
DNA micro-array	Analysis of microbial communities in complex environments	Analyzes a vast amount of genetic information simultaneously	Requires the construction of an array and access to a scanner, Issues with specificity/cross hybridization, Sensitivity and reproducibility can be problematic, Limited by the presence of probes on the array	Guschin et al. (1997)
Raman micro-spectroscopy and Nano-SIMS	Identifying microbial species, linking microbial species to their spatial distribution and their functions, exploration of behavior and functions of unculturable microorganisms	Noninvasive technique to acquire chemical signals from a small volume of samples ($< 1 \mu\text{m}^3$) such as bacteria, Provides cellular information about nucleic acids, proteins, carbohydrates and lipids	Mildly destructive as it removes only the upper one to three atomic layers ($\sim 1 \text{ nm}$) to obtain sufficient sputtered (vaporized) biomass for elemental and isotopic analysis	Huang et al. (2004, 2010); Kuypers and Jorgensen (2007); Oehler et al. (2010)

for unknown genomes. However, as very low annealing temperatures are used, the reproducibility of profiles becomes an issue and it is sensitive to reaction conditions too. Because of the cost effectiveness and ease of use, RAPD has been used extensively in fingerprinting overall microbial community structure and closely related bacterial species and strains (Franklin et al. 1999).

12.3.2 Amplified Fragment Length Polymorphism (AFLP)

AFLPs are PCR-based markers for the rapid screening of genetic diversity. The key feature of AFLP-PCR is its capacity for simultaneous screening of many different DNA regions distributed randomly throughout the genome. In essence, AFLP methods allow PCR amplification to detect polymorphisms of genomic restriction fragments. AFLP markers have proven useful for assessing genetic differences among individuals, populations, and independently evolving lineages, such as species (Vos et al. 1995). The main disadvantage of AFLP-PCR is the difficulty in identifying homologous markers (alleles), rendering this method less useful for studies that require precise assignment of allelic states, such as heterozygosity analysis.

12.3.3 Denaturing/Temperature Gradient Gel Electrophoresis (DGGE/TGGE)

Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TTGE) are based on the separation of PCR amplicons (rDNA fragments) of the same size but with different nucleotide sequences. The separation principle for both methods is applying a linear gradient of DNA denaturing agents (such as a mixture of formamide and urea in DGGE), or temperature (TGGE) on polyacrylamide gels to influence the electrophoretic mobility of partially melted double-stranded DNA. This method is very much preferred for bacterial community analysis. The advantage of PCR-DG/TTGE is that amplicons can be directly extracted from the DG/TTGE acrylamide gel and sequenced. Unidentified profiles that do not match reference profiles can thus be sequenced, compared to publicly available sequence databases and appended to the PCR-DG/TTGE profile database. It has however, a few limitations too, like it is time consuming, can't be reproduced and can be used for only short fragments. In a microbial community investigation, DGGE was applied to soils collected from different agricultural fields in Norway and the USA that were under different agronomic treatments (crop rotation and tillage) (Nakatsua et al. 2000) and the results showed that bacterial diversity was far greater than archaeal diversity except for the PAH-contaminated soil sample. Zhou and Wu (2012) identified changes in structure and composition of bacterial and fungal communities under different concentrations of the autotoxin by using this technique. In addition, molecular fingerprinting of microbial communities demonstrated changes in environmental conditions at a volcanic CO₂ vent (Frerichs et al. 2013).

12.3.4 *Single Strand Conformation Polymorphism (SSCP)*

Single-strand conformation polymorphism is another microbial community analysis technique using either acrylamide gel- or capillary-based automated sequencer, based on the separation of denatured (single-stranded) DNA. Under non-denaturing conditions, single-stranded DNA folds into tertiary structures according to their nucleotide sequences and their physicochemical environment (e.g., temperature and ion strength). This causes differences in electrophoretic mobility in non-denaturing gels. Unlike DGGE, SSCP technology does not require any GC clamped primers, gradient gels, or specialized electrophoretic apparatus; therefore, it is a more simple and straightforward technique than DGGE. This method is advantageous as it helps in identifying new mutants among the community members and also screens potential variants in a sequence. However, when using an automated sequencer, one of the disadvantages of this technique lies in the difficulty of appending new data to an existing database. Further, it is very sensitive, not reproducible and samples presenting unknown profiles cannot be directly sequenced because they are labeled. A major limitation of the SSCP method is the high rate of re-annealing of DNA strands after an initial denaturation during electrophoresis, which can be overcome using a phosphorylated primer during PCR, followed by specific digestion of the phosphorylated strand with lambda exonuclease. SSCP has successfully been employed to differentiate the pure cultures of *Bacillus subtilis*, *Pseudomonas fluorescens*, and *Sinorhizobium meliloti* isolated from soil samples (Schweiger and Tebbe 1998).

12.3.5 *Terminal Restriction Fragment Length Polymorphism (T-RFLP)*

Terminal restriction fragment length polymorphism is based on digestion of fluorescent end-labelled PCR products with restriction endonucleases. Community diversity is estimated by analyzing the size, numbers, and peak heights of resulting T-RFs. Each T-RF is assumed to represent a single OTU or ribotype (Liu et al. 1997). With recent developments in bioinformatics, several Web-based T-RFLP analysis programs have been developed, which enable researchers to rapidly assign putative identities based on a database of fragments produced by known 16S rDNA sequences. The use of a database of T-RF profiles obtained from reference samples allows the identification of the different species of a given community (Dickie et al. 2002). This method has become popular because of its ability to analyze wide array of microbes and high reproducibility. T-RFLP has been used extensively by mycologists to study diverse microbial communities since this method is reportedly more sensitive than PCR-DG/TGGE for fungi (Brodie et al. 2003; Liu et al. 1997). However, some limitations are there due to inefficient restriction enzyme cleavage (Avis et al. 2006) and failure to retrieve sequences. Fierer and Jackson (2006) applied the T-RFLP technique to understand the biogeographical patterns in soil bacterial communities and to investigate the biotic and abiotic factors that shape the composition and diversity of bacterial communities.

12.3.6 *Quantitative Polymerase Chain Reaction (qPCR)*

PCR of soil DNA can be used in a quantitative manner using a so called Taqman or “real-time” approach (Holland et al. 1991). The principle of this method lies in the generation of a fluorescent and detectable signal by exonuclease activity of the polymerase. Signal is produced at each cycle of the PCR reaction. qPCR is currently widely applied to soil-extracted DNA, allowing the quantification of numbers of target genes such as 16S rRNA genes (to quantify soil bacteria) or of functional genes like *amoA* or *nifH*. Although successfully used in many soil studies, qPCR is plagued by some limitations like an inherently biased picture of target gene abundance and inability to detect any gene of the same function with aberrant sequence. However, qPCR may be well employed at the microhabitat/mycosphere level to assess to what extent local conditions affect gene and gene expression levels. Thus, it may be possible to more precisely map microbial diversity and function to soil/mycosphere space. qPCR is also used to determine the total number of copies of 16S rRNA genes of all active bacteria. However, it has a drawback that PCR targeting the genes encoding for 16S rRNA may lead to overestimation of the number of bacteria in a community because some bacteria contain multiple 16S rRNA genes in a single genome (Hoefel et al. 2005). qPCR has also been successfully used in environmental samples for quantitative detection of important physiological groups of bacteria such as ammonia oxidizers, methane oxidizers, and sulfate reducers by targeting *amoA*, *pmoA*, and *dsrA* genes, respectively (Foti et al. 2007).

12.3.7 *Ribosomal Intergenic Spacer Analysis (RISA)*

Ribosomal intergenic spacer analysis (RISA), a commonly used DNA-based community fingerprinting method, is a high-resolution, highly reproducible technique for detecting differences among complex fungal communities (Ranjard et al. 2001). The method has been successfully used to characterize, classify, and type strains, as well as to fingerprint simple communities and mixed populations. It exploits variability in the length of the internal transcribed spacer regions of rRNA genes to sort samples rapidly into operational taxonomic units (OTUs). Although RISA assays a different taxonomic resolution than species level, it is a consistent measure of community composition. Consequently, differences between two OTU assemblages directly reflect changes in species composition (Green et al. 2004). The automated version of RISA is known as ARISA and involves use of a fluorescence-labeled forward primer, and ISR fragments are detected automatically by a laser detector. ARISA allows simultaneous analysis of many samples; however, the technique has been shown to overestimate microbial richness and diversity (Fisher and Triplett 1999). Ranjard et al. (2001) evaluated ARISA to characterize the bacterial communities from four types of soil differing in geographic origins, vegetation cover, and physicochemical properties. ARISA profiles generated from these soils were distinct and contained several diagnostic peaks with respect to size and intensity.

Their results demonstrated that ARISA is a very effective and sensitive method for detecting differences between complex bacterial communities at various spatial scales (between- and within-site variability).

12.3.8 Length Heterogeneity-Polymerase Chain Reaction (LH-PCR)

Length heterogeneity PCR (LH-PCR) analysis is similar to the T-RFLP method except that the latter detects amplicon length variations that are produced after restriction digestion, whereas in LH-PCR different microorganisms are discriminated based on the natural differences between lengths of amplified gene fragments that occur due to mutation (insertion/deletion) within genes (Mills et al. 2007). Amplicon LH-PCR interrogates the hypervariable regions present in 16S rRNA genes and produces a characteristic profile. LH-PCR utilizes a fluorescent dye-labeled forward primer, and a fluorescent internal size standard is run with each sample to measure the amplicon lengths in base pairs. The intensity (height) or area under the peak in the electropherogram is proportional to the relative abundance of that particular amplicon. One advantage of using LH-PCR over the T-RFLP is that the former does not require any restriction digestion and therefore, PCR products can be directly analyzed by a fluorescent detector. The limitations of LH-PCR technique include inability to resolve complex amplicon peaks and underestimation of diversity, as phylogenetically distinct taxa may produce same-length amplicons (Mills et al. 2007). LH-PCR was used in combination with fatty acid methyl ester (FAME) analysis to investigate the microbial communities in soil samples that differed in terms of type and/or crop management practices (Ritchie et al. 2000). LH-PCR results strongly correlated with FAME analysis and were highly reproducible, and successfully discriminated different soil samples.

12.3.9 Fluorescence In Situ Hybridization (FISH)

Nucleic acid hybridization using specific probes is an important qualitative tool in molecular bacterial ecology (Theron and Cloete 2000). These hybridization techniques can be performed on extracted DNA and RNA, or *in situ* hybridization can be conducted at the cellular level. The FISH method has also been used successfully to study the spatial distribution of bacteria in biofilms (Thurnheer et al. 2004). However, with respect to sensitivity, some limitations to the standard FISH method that prevents detection of cells with low ribosome content have been noted. FISH has adopted a tyramine signal amplification technique, which allowed the analysis of slow-growing microorganisms (Pernthaler et al. 2002). Also, a disadvantage of nucleic acid hybridization or FISH is the lack of sensitivity unless sequences are present in high copy number. The FISH method was used to follow the dynamics of bacterial populations in agricultural soils treated with s-triazine herbicides (Caracciolo et al. 2010).

12.3.10 DNA Microarray

The complementary DNA (cDNA) microarray (or microchips) technology has dramatically changed the way gene expression can be assessed (Duggan et al. 1999). Since Guschin et al. (1997) introduced the DNA microarray approach to microbial community analysis, the DNA array-based methods hold great promise for more extensive analyses of microbial communities (Zhou et al. 2003; Bodrossy and Sessitsch 2004; Wagner et al. 2007) and potential applications cover various fields of microbiology, including food science (Bae et al. 2005). However, for this approach to work, each probe must specifically hybridize, under given stringency conditions, to a fully matched DNA target (Valinsky et al. 2002) which has been proven very difficult (Wagner et al. 2007). Moreover, the design and refinement of efficient probes depend on the comprehensiveness and quality of probe target database (Wagner et al. 2007). The low quality of some annotated sequences in the available databases complicates probe design.

12.3.11 Raman Microspectroscopy and NanoSIMS

Single-cell-based technologies such as Raman microspectroscopy (Huang et al. 2004, 2010) and NanoSIMS (Kuypers and Jorgensen 2007; Oehler et al. 2010) are developed to explore the behavior and functions of unculturable microorganisms (Brehm-Stecher and Johnson 2004). Raman microspectroscopy offers a unique opportunity to study microbes at the biochemical level and to manipulate them at single-cell level in their natural habitat. A typical Raman spectrum provides rich cellular information about nucleic acids, proteins, carbohydrates, and lipids. This information provides insights into the molecular composition and structure, and physiological state, which are responsible for differentiation of cell types, nutrient concentrations, and the phenotype changes. Raman microspectroscopy now has been combined with optical tweezers and epifluorescent microscope to develop what is termed as Raman tweezers (Xie and Li 2002; Xie et al. 2005) and combined with FISH techniques to develop into Raman-FISH (Huang et al. 2009). In microbiology research Raman microspectroscopy can be applied for the identification of microbial species using single cell Raman spectra, linking microbial species to their spatial distribution and their functions by Raman-FISH, measuring and manipulating single microbial cells using Raman tweezers, investigating the chemical fingerprinting of microbial cells using surface enhanced Raman scattering (SERS), and measuring the metabolic potential of microbial cells using Raman microspectroscopy. NanoSIMS is one of many new techniques being applied to ancient organic materials in sedimentary rocks. The technique can measure metabolically important elements of carbon, nitrogen, and sulfur (Oehler et al. 2010). Behrens et al. (2008) studied the metabolic interactions of a microbial consortium consisting of a heterotrophic alpha proteobacterium and a filamentous cyanobacterium using enhanced element labeling-catalyzed reporter deposition FISH (EL-FISH) and

NanoSIMS. They found evidence for metabolic interactions by visualizing the fate of substrates labeled with ^{13}C carbon and ^{15}N nitrogen, while individual cells were identified simultaneously by halogen labeling via EL-FISH. Similarly, Dekas and Orphan (2011) identified diazotrophic microorganisms in marine sediments using FISH coupled with NanoSIMS (FISH–NanoSIMS). In the future, NanoSIMS will facilitate further studies of the ecophysiology of known and uncultured microorganisms in complex environments and communities.

These culture-independent methods have many advantages over culture-dependent methods. These methods are rapid and allow simultaneous analyses of a large number of samples. As for example, methods like qPCR and RISA/ARISA are quick, accurate and highly sensitive whereas some others like DGGE/TGGE, T-RFLP, etc. are sensitive to variation in DNA sequences, highly reproducible and can be used for analyses of large amount of unknown genome. The efficiency and reliability of these methods are far better than the culture-dependent ones and this can be attributed to the conserved nature of the 16S rRNA gene on which these methods are dependent. They can also help in identifying new mutants and store data and compare between different samples.

These culture-independent techniques, however, are not without limitations. The limitations include difficulty in accessing every genotype from the community, poor DNA extraction yield, PCR inhibition due to various by-products or substances coming out from the environment itself. Further, techniques like DGGE/TGGE, SSCP and T-RFLP have limitations in terms of resolution. They can generate patterns in which different genotypes group together due to co-migration/co-elution (Ogier et al. 2002; Feurer et al. 2004a; Lafarge et al. 2004; Delbès et al. 2007; El-Baradei et al. 2007) or ‘co-hybridization’ (Wagner et al. 2007). The techniques like FISH, qPCR and microarray are based only on known samples and will not detect those that do not have corresponding probes on the array. Another limitation of gel/capillary migration-based methods is obtaining profiles in which the less-common amplified sequences cannot be distinguished from background noise (Feurer et al. 2004a; Callon et al. 2006). This problem increases when the community is highly diversified. Authors like Ercolini et al. (2001), Feurer et al. (2004a, b), Florez and Mayo (2006) and Delbès et al. (2007) are of the opinion that culture-independent methods regularly fail to identify species obtained using culture-dependent methods. These two types of methods reveal different images of the same community. They, therefore, suggest that it would always be wise to use combination of culture-dependent and culture-independent methods to obtain a more accurate view of the structure of the microbial community.

12.4 Metagenomic Approach

The limitations of culture independent techniques have introduced a new approach known as “*Metagenomics*”. It is the study of genomic material obtained directly from the environment. It enables characterization of whole communities with no

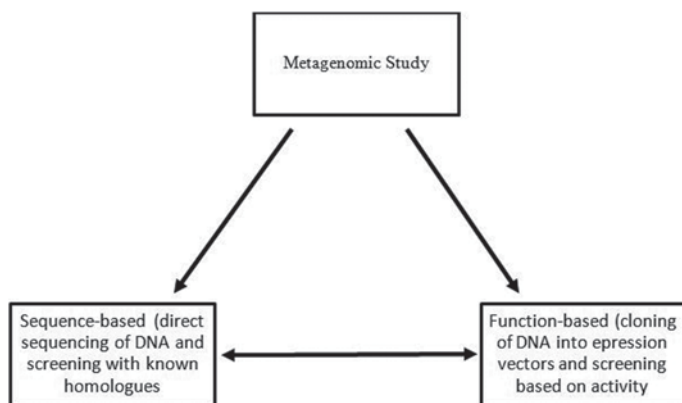


Fig. 12.2 Metagenomic approaches for microbial diversity studies

prior knowledge of the organisms or specific genes present and provides information on functional genes, as well as, taxonomy and diversity. Metagenomics is a fast growing and diverse field within biological sciences directed at obtaining knowledge on genomes of environmental microbes, as well as of entire microbial communities, omitting the cultivation step (Handelsman et al. 1998). Other terms used to describe this methodology are environmental genomics, ecogenomics, community genomics and these are all interchangeable.

Metagenomic techniques are based on the concept that the entire genetic composition of environmental microbial communities could be sequenced in the same way as the whole genome of a pure bacterial culture using different molecular methods. It provides phylogenetic and functional diversity of uncultured microorganisms across number of habitats like soil, phyllosphere, ocean and acid mine drainage (Handelsman 2004).

The metagenomical studies are based on two types of approaches viz. function-based and sequence-based (Fig. 12.2). The two approaches are not mutually exclusive. Instead, their co-existence reflects both the history of metagenomics and the interdependence of the two methodologies. The function-based approach requires cloning of environmental DNA into expression vectors and propagating them in appropriate hosts, followed by screening for desirable activity (Craig et al. 2010; Schmeisser et al. 2007; Streit et al. 2004). After an active clone is identified (referred to as a hit), the clone is sequenced, the gene of interest, its product and their biotechnological potential is explored. The sequence-based metagenomic approach requires prior knowledge on proteins possessing the activity of interest, and the screening is performed toward the genes that are predicted to encode proteins with specific sequences/folds/domains indicative of their functionality. The field of metagenomics has been transformed by the application of a whole genome shotgun (WGS) sequencing technology and revolutionized the field of microbial genomics (Fleischmann et al. 1995). The advances in next-generation (ultra-high throughput)

sequencing technologies like Roche 454, SOLEXA/ILLUMINA etc. have made DNA sequencing quite accessible, fast and cost-effective for the researchers. These developments have further strengthened the metagenomic studies for diversity analysis of microbes.

Current technologies in the field can broadly be classified in two categories (a) sampling and sequencing, and (b) metagenome analysis. Sampling methods depend on the niche and habitat where the microorganisms are to be studied (Abe et al. 2005). Several metagenomic sequencing projects such as acid mine drainage (AMD) biofilm, human gut microbiome (HGM) have successfully been implemented since 2004 (Yang et al. 2009) (Table 12.2). The methodology used in metagenomic analysis includes many steps starting with extraction of DNA from soil samples, selection of suitable host and vector for cloning or direct sequencing of the DNA, construction of metagenomic library to amplification of DNA using universal primers to generate 16S rRNA gene library and analysis of cloned fragments using sequence-based or functional screening approaches (Fig. 12.3). The entire methodology has been discussed below.

12.4.1 DNA Extraction

Understanding soil microbial diversity through metagenome approaches requires sufficient quantity of high quality DNA in order to construct cloned libraries (Vogel et al. 2009). The DNA extraction is the key step in metagenomics and the extraction process may be direct and indirect based on whether the cells have been separated from the soil sample prior to the cell lysis process. Delmont et al. (2011) compared the two extraction methods and concluded that though the indirect approach is time-consuming and requires specific materials like extraction chemicals, enzymes etc. and sufficient quantity of soil as compared to direct method yet it has advantages like it yields less sheared DNA and reduced proportion of eukaryotic sequences. The different methods employed for DNA extraction involve *in situ* lysis and extraction by physical, chemical or enzymatic agents, extraction by sonication or bead beating or a combination of both and so on (Fig. 12.4).

When DNA is extracted from soil, humic acids also get extracted as contaminants. Therefore, it is necessary to evaluate the purity of the extracted environmental DNA (eDNA). For this purpose absorbance ratios at 260/230 nm (DNA/humic acid) and 260/280 nm (DNA/protein) are determined. A ratio in the range of 1.8–2 indicates good quality of DNA. The quality and size of the extracted DNA samples can also be estimated on 0.8 % agarose gels using TAE as electrophoresis buffer and staining with ethidium bromide. Such good quality DNAs once obtained are then used in different metagenomic approaches such as cloning, library construction, direct DNA sequencing, amplification of target DNA regions in PCR, etc.

Table 12.2 List of metagenomic works carried out in various habitats

Metagenome study	Ecosystem studied	Significant findings	References
Marine microbial communities from the lost city hydrothermal field	Marine	A remarkable abundance and diversity of genes potentially involved in lateral gene transfer (LGT)	Brazelton and Baross (2009)
Acid mine drainage (AMD) euryarchaeal community from UBA site, at Richmond mine, Iron Mountain, CA	Freshwater	Proteomics results reveal a genome shaped by recombination involving chromosomal regions of tens to hundreds of kilobases long that are derived from two closely related bacterial populations. Inter-population genetic exchange was confirmed by multilocus sequence typing of isolates and of uncultivated natural consortia. The findings suggest that exchange of large blocks of gene variants is crucial for the adaptation to specific ecological niches within the very acidic, metal-rich environment	Lo et al. (2007)
Fossil microbial Community from Whale Fall, Santa Cruz Basin of the Pacific Ocean	Zoolites (fossil)	Characterizes and compares the metabolic capabilities of terrestrial and marine microbial communities using largely unassembled sequence data obtained by shotgun sequencing DNA isolated from the various environments. Quantitative gene content analysis reveals habitat-specific fingerprints that reflect known characteristics of the sampled environments	Tringe et al. (2005)
Human skin microbiome from ten healthy volunteers, NISC	Human skin	The analysis of 16S ribosomal RNA gene sequences obtained from 20 distinct skin sites of healthy humans revealed that physiologically comparable sites harbor similar bacterial communities. The complexity and stability of the microbial community are dependent on the specific characteristics of the skin site	Grice et al. (2009)
Soil microbial communities from Waseca County, Minnesota Farm	Soil	This study characterize and compare the metabolic capabilities of terrestrial and marine microbial communities using largely unassembled sequence data obtained by shotgun sequencing DNA isolated from the various environments. Quantitative gene content analysis reveals habitat-specific fingerprints that reflect known characteristics of the sampled environments	Tringe et al. (2005)

Table 12.2 (continued)

Metagenome study	Ecosystem studied	Significant findings	References
Soil microbial communities from Alaska	Soil	This study reports that this soil is a reservoir for beta-lactamases that function in <i>Escherichia coli</i> , including divergent beta-lactamases and the first bifunctional beta-lactamase. The findings further suggest that even in the absence of selective pressure imposed by anthropogenic activity, the soil microbial community in an unpolluted site harbors unique and ancient beta-lactam resistance determinants. Moreover, despite their evolutionary distance from previously known genes, the Alaskan beta-lactamases confer resistance on <i>E. coli</i> without manipulating its gene expression machinery, demonstrating the potential for soil resistance genes to compromise human health, if transferred to pathogens	Allen et al. (2009)
Chicken cecal microbiome from University of Illinois, Urbana-Champaign	Bird	This analysis has demonstrated that mobile DNA elements are a major functional component of cecal microbiomes, thus contributing to horizontal gene transfer and functional microbiome evolution. Moreover, the metavirulomes of these microbiomes appear to associate by host environment. These data have implications for defining core and variable microbiome content in a host species. Furthermore, this suggests that the evolution of host specific metavirulomes is a contributing factor in disease resistance to zoonotic pathogens	Qu et al. (2008)
Human fecal microbial communities from the University of Osaka, Japan, of patients with bacterial infections	Human digestive system	This study involves a directed survey of the human subgingival crevice and isolation of bacteria having rod-like morphology. Several isolated microbes had a 16S rRNA sequence that placed them in candidate phylum TM7, which has no cultivated or sequenced members. Genome amplification from individual TM7 cells led to sequence and assemble >1,000 genes, providing insight into the physiology of members of this phylum	Nakamura et al. (2008)

Table 12.2 (continued)

Metagenome study	Ecosystem studied	Significant findings	References
Marine plankton microbial communities from the deep Mediterranean sea	Marine	The comparison of metagenomic libraries from the deep Mediterranean and the Pacific ALOHA water column showed that bathypelagic Mediterranean communities resemble more mesopelagic communities in the Pacific, and suggests that, in the absence of light, temperature is a major stratifying factor in the oceanic water column, overriding pressure at least over 4000 m deep. Several chemolithotrophic metabolic pathways could supplement organic matter degradation in this most depleted habitat.	Martin-Cuadrado et al. (2007)
Fossil microbiome from Neanderthal (JGI)	Soil (fossil)	The study showed that the Neanderthal and human genomes are at least 99.5 % identical leading to development and successful implementation of a targeted method for recovering specific ancient DNA sequences from metagenomic libraries. This initial analysis of the Neanderthal genome advanced the understanding of the evolutionary relationship of <i>Homo sapiens</i> and <i>Homo neanderthalensis</i> and signifies the dawn of Neanderthal genomics	Noonan et al. (2006)

N.B. Information on various metagenomic works around the world can be obtained from the website GOLD (www.genomesonline.org)

12.4.2 Cloning and Construction of Metagenomic Library

Various vectors are now being used to prepare a clone library. A suitable vector is selected on the basis of cloning strategy as well as on the size of the metagenomic DNA fragments to be cloned. The libraries with small inserts (upto a few kb) are constructed using plasmid vectors, libraries with medium inserts (upto several tens of kb) use cosmids or fosmids, and libraries with larger inserts (upto 100–200 kb) use bacterial artificial chromosome (BAC) vectors. Usually *E. coli* based systems are used as expression systems for metagenomic libraries constructed by using plasmids as standard cloning procedures. Plasmids such as pBluescript SK+ and pCR-XL-TOPO have been used as cloning vectors (Henne et al. 2000; Knietsch et al. 2003) for expression of small genes encoding enzymes like lipase and protease. The fosmid and cosmid vectors are usually used for screening of functional genes like those of chitinase and amylase (Brady and Clardy 2000; Entcheva et al.

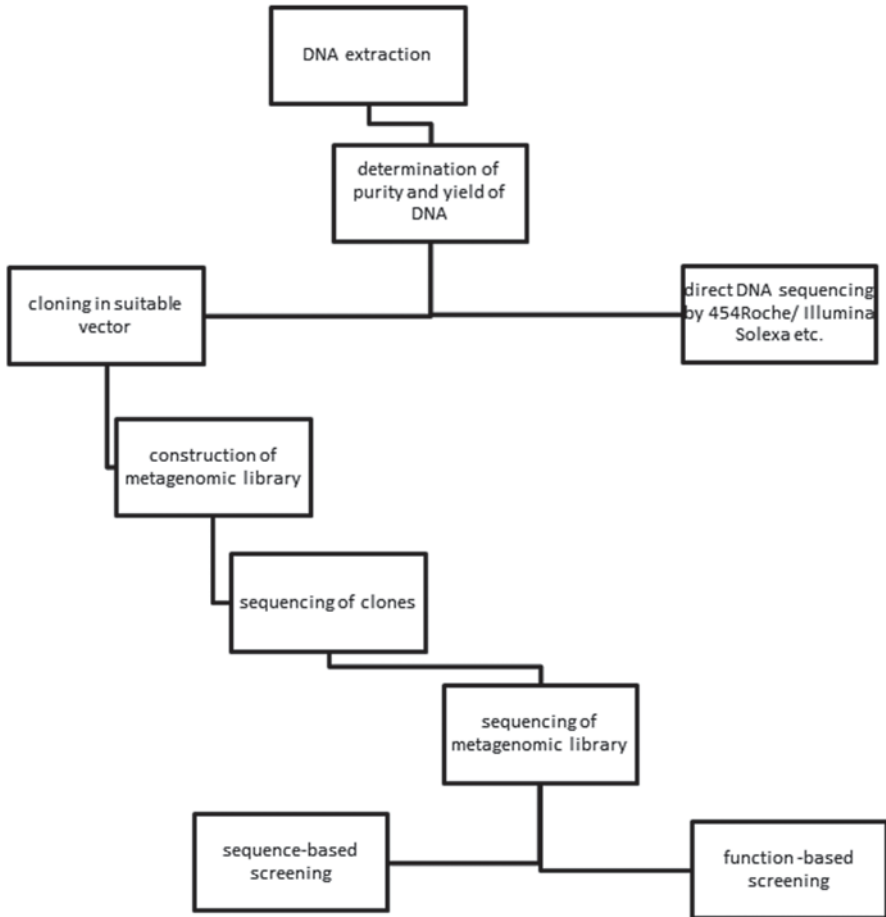


Fig. 12.3 The entire method of metagenomic sequences

2001). The BAC vectors are proving to be very important for cloning of large DNA fragments and mapping of complex genomes (Shizuya et al. 1992) like those of environmental DNA. Several shuttle vectors have also been developed now-a-days for heterologous DNA expression in more than one host. For example, the pPAC-S2 vector is an *E. coli-Streptomyces* artificial chromosome (ESAC) shuttle vector which has been used to clone and express soil metagenomic DNA of upto 100 kb in *E. coli* and *S. lividans* hosts (Berry et al. 2003).

The size of the metagenomic library i.e., the number of clones that are required to form the library can be estimated, assuming that species are equally represented in the metagenome and can be given by:

$$N = \ln(1 - P) / \ln(1 - f)$$

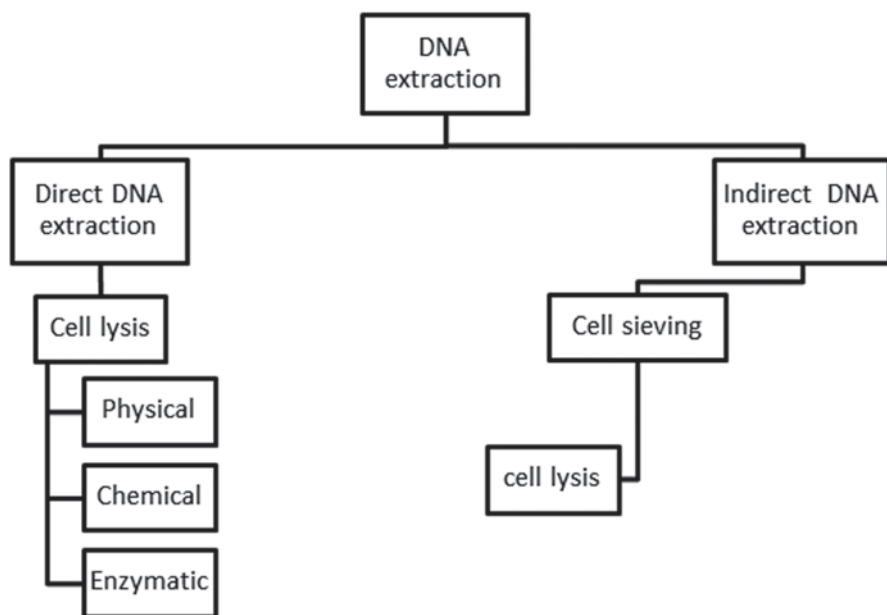


Fig. 12.4 Different DNA extraction methods

where P is the desired probability expressed as a fraction, and f is the proportion of the genomes contained in a single clone (Clarke and Carbon 1976). However, the library has to be normalized for even representation of all species in the soil.

Once the clone library becomes ready, then sequencing of the cloned DNA inserts is done for further screening of the metagenomic library. The metagenomic library can be screened either by sequence-based screening or by functional expression screening. The sequencing of DNA inserts can be done by either conventional Sanger's sequencer or nexgen sequencer. The sequence-based screening methods include analyses via random sequencing, end-sequencing as well as transposon facilitated sequencing. Phylogenetic screening using 16S rRNA gene PCR amplification has been used to analyze the diversity of metagenomics libraries prepared from selected soil biotopes (Rondon et al. 2000; Quaiser et al. 2003). Functional screening is achieved through selection for a phenotype, which can be an enzymatic activity or a metabolite produced by expressed recombinant clones. For example, the clones so obtained are screened for a specific trait like production of an enzyme or pigment. Functional screening relies on accessibility of the substrate along with correct transcription, translation and folding of the gene product (protein). The phenotypic selection is done by high-pressure liquid chromatography (HPLC) or mass spectrometry (MS) or thin layer chromatography (TLC).

12.4.3 Direct DNA Sequencing

The extracted DNA can be directly sequenced too, without undergoing the process of library construction. For this now a days next- generation sequencing (NGS) technologies are preferred over the classical Sanger sequencing technology which is a labor intensive and expensive (Thomas et al. 2012). These high-throughput sequencing approaches are now increasingly being used for estimates of microbial diversity. The most widely-used platforms for massive parallel sequencing for assessing soil microbial diversity are Roche 454 Genome Sequencer (Roche Diagnostics Corp., Branford, CT, USA), HiSeq 2000 (Illumina Inc., San Diego, CA, USA), AB SOLiD™ System (Life Technologies Corp., Carlsbad, CA, USA), Life/APG, and HeliScope/Helicos BioSciences. The NGS technologies like 454/Roche and the Illumina/Solexa systems are now being extensively used for metagenomic samples because of their inexpensiveness and production of an average read length (600–800 bp) which is long enough to cause only minor loss in the number of reads that can be annotated (Thomas et al. 2012).

The advantage of using the NGS techniques is that multiple environmental samples can be combined in a single run. Further these techniques are rapid methods for assessing biodiversity and abundance of many species/organizational taxonomic units simultaneously and at a considerable depth compared to the methods that have been available so far. As for example, Lauber et al. (2009) got an enormous diversity of soil microbial communities with an average of at least 1000 species per soil sample by using 454/Roche pyrosequencing technique. This result suggested that soil bacterial communities are extremely diverse and contain a large “rare biosphere” represented by an enormous number of low-abundance unique taxa. Such studies highlight the importance of large-scale sequencing techniques in investigating the highly diverse soil microbial communities. The limitations of these techniques however, lies in generation of a large data which has to be analyzed by computational softwares only and can be accepted valid only after doing statistical analysis (Rincon-Florez et al. 2013).

12.4.4 Amplification of Targeted DNA Region (GENE)

The metagenomic DNA can also be directly used for the amplification of any targeted genes like 16S rRNA, amoA, nifH with their known primers sequences depending on the objectives of the study.

12.4.5 Sequence Analysis and Annotation

After the amplification of the targeted region, the required DNA is sequenced mostly by NGS techniques or sometimes by the classical Sanger technique. For the analysis of data, at first individual sequences are assigned to their corresponding samples

and then correction of errors introduced by PCR (chimers) and sequencing is done. It is followed by clustering of sequences at a specified similarity percentage to form taxonomic groups or operational taxonomic units (OTUs). Metagenomic datasets are most often compared to known sequences using BLAST. Statistical tools enable us to rapidly and accurately compare large datasets generated from complex microbial communities and to identify features that distinguish them. For example, we can compare the taxonomic composition of the microbial communities present in two habitats. The metagenomic data are presented in a tabular form, where the columns represent samples and the rows indicate either a taxonomic group or a gene function and the fields containing abundance or presence/absence of data. Some of the commonly used tools for statistical analyses are the Primer-E package (Clarke and Ainsworth 1993), Metastats (White et al. 2009) which is a web-based tool, Shotgun-Functionalize R package which is used for assessing functional differences between samples, both for individual genes and for entire pathways. METAGEN assist, BiodiversityR, VEGAN R, SPSS and POPGene 32 softwares are also used for statistical analysis.

For the analysis of the obtained data, the steps to be followed are assembly, taxonomic binning, functional analysis and phylogenetic analysis.

12.4.5.1 Assembly

For the data analysis, at first, assembly of short read fragments are performed to obtain longer genomic contigs and the assembly can be done either by using reference-based strategy or by de novo strategy. The factors to be considered while assembling metagenomic data depends on the length of the sequencing reads used to generate the metagenomic dataset and the reduction of data-processing requirements by the assembled dataset.

12.4.5.2 Taxonomic Binning

The taxonomic characterization of DNA fragments (reads) obtained from sequencing a sample of mixed species is usually referred as “binning”. This involves sorting of DNA into groups that represent an individual genome or genomes from closely related organisms. Among the computational tools recently developed for metagenomic sequence analysis, binning tools attempt to classify all (or most) of the sequences in a metagenomic dataset into different bins (i.e., species), based on various DNA composition patterns (e.g., the tetramer frequencies) of various genomes.

12.4.5.3 Functional Analysis

There are mainly two broad objectives of the functional analysis viz. to determine what are the functional and metabolic repertoires of the different community

members that enable them to exert different effects and to identify the variations, if any, within the functional compositions of the different communities (Prakash and Taylor 2012). According to Prakash and Taylor (2012), functional analysis of the metagenomic data plays a central role in diversity studies by providing important clues about the genes responsible for the functional and metabolic diversity, as well as variation within a microbial community. However, functional analysis is limited as it provides only partial information of the microbial community in an environment i.e., only a mixture of fragmented sequences from the community members, and mostly from dominant members of the environment are obtained and this results into analysis of genes and their products present on these fragments only. However, a combination with other approaches like metatranscriptomics, metabolomics, metalipidomics etc. will help to gain further insights about the diversity of a given community. Some of the commonly used tools for functional analysis are IMG/M, METAREP, CAMERA, RAMMCP, MG-RAST, MEGAN4, CoMet, WebMGA etc.

12.4.5.4 Phylogenetic Analysis

The success of any of these methods for community characterization relies on a suitable phylogenetic analysis because many of the organisms that are likely to be described from soil communities have not been reported previously. A number of phylogenetic methods like parsimony, maximum likelihood (ML), and MCMC-based Bayesian inference have been utilized in studies of microbial ecology (Woese 1987). For microorganisms, molecular data are of greatest importance because microorganisms such as bacteria are morphologically primitive and do not have the diversity of form to make morphological characteristics useful in establishing phylogenies. Alongwith taxonomy, Phylogenetic analyses are important in identifying similarities between organisms, in order to understand the physiology and ecology of as yet non-culturable species. Phylogenetic analyses have a major drawback. It has been observed that though an analysis based on a single type of molecule results in a close relationship between taxa but it does not necessarily mean that another, equally suitable molecule will support these results, although this often occurs (Olsen and Woese 1993). When based on a limited set of taxonomic criteria, it is difficult to say with certainty whether or not those criteria can resolve an unknown microorganism from other known microorganisms. Therefore, microbial phylogenies should be interpreted with caution when used in soil microbial community analyses.

Metagenomics has now become one of the primary tools for understanding the biochemical roles of uncultured microorganisms and their interaction with other biotic and abiotic factors as it avoids the biases of culture-independent techniques. Further, it has also shed light on various other aspects of a microbial community like its genomic organization and traits that are acquired from distinctly related taxa through horizontal gene transfers (Handelsman 2004). The environmental metagenomics have also led to the discovery of new enzymes and antibiotics (Rondon et al. 2000; Riesenfeld et al. 2004).

12.5 Culture-Independent Methods and Metagenomics in Conservation of Microbial Diversity

Study of microbial diversity is fundamental for the maintenance and conservation of global genetic resources. As extreme environments are explored, the richness of microbial diversity is increasingly evident. Measures are taken to estimate, record, and conserve microbial diversity, not only to sustain human health but also to enrich the human condition globally through wise use and conservation of genetic resources of the microbial world. The conservation and utilization of biological diversity requires comprehensive knowledge about the species distribution so as to keep the ecological balance in an environment (Das et al. 2006). Advances in molecular techniques have given us a glimpse of the tremendous diversity present within the microbial world, but significant work remains to be done in order to understand the ecological and evolutionary dynamics that can account for the origin, maintenance, and distribution of that diversity (Cohan and Perry 2007).

Culture-independent methods provide a more accurate picture of microbial communities because most microbes from soil are not culturable. Further, as these techniques are based on genes which are conserved in nature, hence, the data obtained are more robust and reliable. The culture-independent methods along with metagenomic approach has provided a vast array of microbial diversity from various habitats like soil, water etc. The large-scale sequencing technologies have allowed us to investigate deeper and deeper layers of the microbial communities and are vital in presenting an unbiased view of phylogenetic composition and functional diversity of environmental microbial communities (Zwolinski 2007). Metagenomics approaches have been applied to understand the structure (gene/species richness and distribution) and the functional (metabolic) potential of environmental microbial communities. The environmental metagenomic libraries have proved to be great resources for new microbial enzymes and antibiotics with potential application in biotechnology, medicine and industry (Riesenfeld et al. 2004; Rondon et al. 2000). Especially, the microbial metagenomes derived from soil have proven to be a rich source for the discovery of novel antibiotics (e.g., turbomycin, terragine), enzymes (e.g., cellulases, lipases, amylases) and proteins (e.g., antiporters) (Rondon et al. 2000). Further, metagenomics has provided many tools for assessing species diversity using different sequence markers for OTU identification. The use of non-rDNA phylogenetic markers like various housekeeping genes have showed that certain microbial communities evolve faster than others.

As the culture-independent studies and metagenomic approach has led to identification of many unculturable microbes as well as new species and novel genes, hence they can be considered as revolutionizing the microbial diversity studies. These technologies have helped researchers to go deeper into genome research and conservation strategies. The analysis of microbial diversity with culture-independent studies is aiding in conserving the microbial gene pools for useful biotechnological applications. Further, the microbial gene pool obtained via culture-independent studies and metagenomics will enable us to have knowledge on indigenous microbial population of a certain ecosystem and thereby we can conserve these

microbes *in situ* by conserving that particular ecosystem. Conservation of microbes is important as they play an important role in all ecosystem functions like biogeochemical cycles, soil formation, plant nutrition and so on. Both *in situ* and *ex situ* conservation are given equal importance now.

In situ preservation involves on site conservation of the microbial flora involving the conservation of the ecosystems and natural habitats and the maintenance as well as recovery of viable populations of species in their natural surroundings and in case of the domesticated or cultivated species, in surroundings where they have developed their distinctive properties. However, because of uncertainties in *in situ* conservation of microorganisms like contamination of cultures, *ex situ* preservation in the form of gene banks, culture collection centres and microbial resource centers is now preferred. These centers being repository for microbial isolates is helping to save time and money, which would have gone into reisolation procedures of the microbes. Species and strains can be conserved by isolation and culturing *ex situ*, in which they are maintained as a specific type strain in a culture collection (Supardiyo and Smith 1997; Arora et al. 2005), but this species-specific conservation is difficult to achieve in the natural environment and may be one reason for the apparent impracticability of including microorganisms in conservation agenda (Cockell 2009). The generally large population size of most species and their ubiquity further reduces the perceived need to protect them and focuses attention instead on their habitats (Gerhardson and Wright 2002). The small scale of the organisms contributes to other reasons for their omission from conservation goals, including their general lack of macroscopically visible qualities that can be appreciated by the general public, which provides a powerful impetus for the conservation of many animals and plants.

Conserving microbial diversity will often, in a practical sense, equate to the conservation of the ecosystem microbial gene pool. From a pragmatic point of view, the conservation of the gene pool and microbial diversity itself equates to the conservation of the physical and chemical conditions within an environment that best support the indigenous microbiota. Conservation efforts for microorganisms could be achieved by focusing on the protection of habitats, although the protection of habitats is itself motivated by the fact that they contain microbial transformations of importance, so a more direct conservation ethic would draw attention to habitats and their microorganisms. Once ecosystem diversity and health have been secured, it would then be possible to contemplate conservation of microorganisms, from specific species to phyla, where such specific identification can actually be achieved. Microbial conservation efforts would also focus, however, on preserving these taxa in their natural habitat, in particular because the same environments may harbor similar but as yet undiscovered products. Some polluted environments harbor microorganisms that can adapt to the extreme conditions. Sites polluted by heavy metals such as arsenic, chromium or uranium host organisms with unique genes and biochemical pathways (Stierle et al. 2007). Investigating these organisms is not only beneficial for understanding the effects of pollution; it can also yield insights into natural toxic environments. Microorganisms that play a role in global biogeochemical cycles are a high priority for conservation because of the importance of these cycles to the rest of the biosphere. These communities include ocean

phytoplankton and ocean sediment communities, many of which are threatened by anthropogenic activities.

In India conservation work has been carried out by Ministry of Environment and Forestry and the Ministry of Science and Technology that includes various departments such as the Department of Agriculture Research and Education, Indian Council of Forestry Research and Education, Department of Biotechnology etc. The level of the Microbial Type Culture Collection section of IMTECH, Chandigarh has now been upgraded to an International Depository Authority (IDA) and it involves the culture collection and maintenance as well as distribution of pure cultures internationally. Another microbial culture collection centre named as Microbial Culture Collection (MCC) is in Pune under the aegis of National Centre for Cell Science, Department of Biotechnology, Govt. of India. Seeing the potential of north-east India as a biodiversity hotspot, a culture collection centre has been developed in Imphal, (Manipur) and named as Institute of Bioresources and Sustainable Development.

In brief we can say that the knowledge on microbial diversity and strategies for their conservation is going hand in hand and its credit goes to the development of various culture- independent studies including metagenomics.

12.6 Future Prospect of Microbial Diversity Studies

The scientific community is now facing challenges of human welfare like food, security, medical, energy sustainability and climate change. Researchers have been motivated to develop novel approaches towards harnessing the potential of various microbes for sustainability of these welfare aspects. The soil being one of the richest sources of microorganisms is now taken into focus and exploration of soil microbial diversity is taking place. Hence new technologies and new projects have been developed to face challenges posed by such a complex and diverse environment. The high-throughput culture- independent techniques will help in dealing with all emerging data in this regard in a reasonable timeframe. However, the large number of organisms involved and the highly dynamic nature of the soil itself makes it challenging to provide the level of detail afforded by molecular techniques. The future of soil microbial diversity studies seems to be bright as it will find place in various fields like agriculture, food, industry etc. where these studies will be applied to get novel genes/products which will be beneficial to us.

12.7 Conclusion

One of the primary challenges in modern microbial ecology is effectively and accurately assessing total microbial diversity, particularly with regard to detection of uncultivable and fastidious microbial species and those present in low abundance. Metagenomics has changed the way microbiologists approach many problems, re-

defined the concept of a genome, and accelerated the rate of gene discovery. The potential for application of metagenomics to biotechnology seems endless. Functional screens have identified new enzymes and antibiotics and other reagents in libraries from diverse environments. The management and analysis of large libraries have been intensified by advances in bioinformatics tools. These tools analyze vast sequence databases and reassemble multiple genomes rapidly and provide affordable gene chips for library profiling and readily distinguish clones expressing genes from those clones that are silent.

Microbiology has long relied on diverse methods for analysis, and metagenomics can provide the tools to balance the abundance of knowledge attained from culturing with an understanding of the uncultured majority of microbial life. Myriad environments on Earth have not been studied with culture-independent methods other than PCR-based 16S rRNA gene analysis, and hence, other methods like Raman microspectroscopy and NanoSIMS etc. can be used for further analysis. Metagenomics may further our understanding of many of the exotic and familiar habitats that are attracting the attention of microbial ecologists, including deep sea thermal vents; acidic hot springs; permafrost, temperate, desert, and cold soils; Antarctic frozen lakes; and eukaryotic host organs—the human mouth and gut, termite and caterpillar guts, plant rhizospheres and phyllospheres, and fungi in lichen symbioses. With improved methods for analysis, funding stimulated by recent triumphs in the field, and attraction of diverse scientists to identify new problems and solve old ones, metagenomics will expand and continue to enrich our understanding of microorganisms.

Understanding the functional roles of uncultured organisms still remains a daunting task, as most of the genes identified have no homologous representatives in databases. Although considerable progress has been made in the characterization of microbial communities by the application of metagenomic approaches, many technical challenges remain including DNA, RNA, and protein extraction from environmental samples, mRNA instability, and low abundance of certain gene transcripts in total RNA. The next-generation sequencing techniques are still developing, and many technological innovations particularly tuned for environmental samples are expected in these techniques. Development in bioinformatics tools is also needed for evaluating the tremendous amount of information generated through whole-genome analysis and metagenomic approaches. Quantitative assessment of microbial communities is the greatest challenge due to significant biases associated with nucleic acid isolation and PCR and requires more advanced DNA/RNA extraction techniques for environmental samples.

All of the molecular approaches available for community structure and function analysis have advantages and limitations associated with them, and none provides complete access to the genetic and functional diversity of complex microbial communities. A combination of several techniques should be applied to interrogate the diversity, function, and ecology of microorganisms. Culture-based and culture-independent molecular techniques are neither contradictory nor excluding and should be considered as complementary. An interdisciplinary systems approach embracing several “omics” technologies to reveal the interactions between genes, proteins, and

environmental factors will be needed to provide new insights into environmental microbiology. Development of multi-“omics” approaches will be a high-priority area of research in the coming years. Conversely, metagenome-sequencing will give us complete information of biodiversity in the environment, while it requires huge budget and time.

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Chapter 13

The Importance and Application of Bacterial Diversity in Sustainable Agricultural Crop Production Ecosystems

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Abstract Soil inhabiting bacteria are integral to global biogeochemical cycles and influence nutrient cycling and mineral solubilization important to soil health, and crop productivity. Soil bacteria directly impact plant fitness as pathogens, beneficial mutualists, and indirectly as decomposers, or through antagonistic activity against plant pathogens. Moreover, such beneficial bacteria have the capacity to produce plant hormones and induce systemic disease resistance responses in plants. This chapter discusses bacterial diversity and its role in sustainable agriculture in crop ecosystems. The chapter also addresses the potential role of plant growth promoting rhizobacteria (PGPR), biological control agents (BCAs) and the significance of bacterial community diversity associated with soil borne plant disease suppression in sustainable agricultural crop production. The influence of herbicide resistant crop rotation systems on soil bacterial diversity is discussed. Current culture-independent approaches to study bacterial diversity, and directions for future applied research in agricultural production systems are also discussed.

13.1 Introduction

Although biological diversity (or biodiversity) can be described as “the total diversity of life on earth”, a disproportionate scientific emphasis has been placed on understanding plant and animal versus bacterial diversity. Far less is known about bacterial diversity despite the integral role it plays in the health and functioning of ecosystems via the direct and indirect influence on its abiotic and biotic components (Michelsen et al. 1999; Bohlen et al. 2001; Shively et al. 2001; Klironomos 2002; Zak et al. 2003). Moreover the soil micro-flora encompass a wealth of functional diversity (Maire et al. 1999; Dunbar et al. 2002; Ibekwe et al. 2002; Zhou et al. 2002; Fierer et al. 2003; Horner-Devine et al. 2003; Thirup et al. 2003) and is hypothesized to be a major repository of as yet uncultivated biodiversity (Tiedje et al. 1999; De-Long and Pace 2001; Torsvik and Ovreas 2002) with the potential to be mined for practical tools to enhance sustainability in current crop production systems.

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The rhizosphere is defined as the soil influenced by, or in association with plant roots and plant-produced material. Moreover the rhizosphere includes the region of soil bound by plant roots, often extending a few mm from the root surface. Rhizosphere soil is much richer in bacteria and biological activity than the surrounding bulk soil (Hiltner 1904) as cited by Bringham et al. (2001). The structure of rhizobacterial communities is determined by the plant species (Goddard et al. 2001; Smalla et al. 2001; Marschner et al. 2004), and differences in the composition and amounts of root exudates probably account for the differences in microbial populations. Recent studies suggest that an individual gram of soil can contain between 2000 and 10^6 distinct genomes (Torsvik and Ovreas 2002; Gans et al. 2005) and more than 4000 microbial species are present per gram of rhizosphere soil (Montesinos 2003). Plant exudates such as amino acids and sugars provide a rich source of energy and nutrients for the bacteria in rhizosphere, resulting in more microbial populations in this region of the soil (Haas and Dèfago 2005). Within the rhizosphere soil matrix resides microbial communities that can be defined by function and biological characteristics, such as PGPR, antagonistic microorganisms, endophytes, mycorrhizae, as well as deleterious microorganisms. A better understanding of improved sustainability in agricultural crop production systems that involves plant host, environmental, soil characteristics, pathogens, and other non-pathogenic or beneficial microorganisms is essential.

13.2 Importance and Application of Bacterial Diversity in Agriculture

Rhizodeposits released from plant roots (e.g., nutrients, exudates, border cells and mucilage) attract bacteria to the rhizosphere. Thus, the rhizosphere and the whole of its associated bacteria (microbiome) are widely considered the apex for understanding diversity of bacteria essential for the maintenance of crop and soil health. Moreover, the rhizosphere microbiome is widely considered the most complex ecosystem in nature (Hartmann et al. 2004; Pierret et al. 2007; Jones and Hinsinger 2008; Hinsinger et al. 2009; Raaijmakers et al. 2009; Mendes et al. 2013). The relative abundances of 147 of the 1917 bacterial taxa detected in the rhizosphere of *Avena fatua* (common wild oat) were found to be significantly different from the bacterial taxa in the bulk soil, with most of the rhizosphere species belonging to the Actinobacteria, Firmicutes, and Proteobacteria (DeAngelis et al. 2008). Similarly culture-independent approaches also revealed that most species of rhizosphere bacteria belong to the Actinobacteria, Firmicutes, and Proteobacteria in both agricultural crop production and native prairie ecosystems (Rosenzweig et al. 2012, 2013).

Within the Actinobacteria, Firmicutes, and Proteobacteria representatives of the genera *Streptomyces*, *Bacillus* and *Pseudomonas* respectively, are known to be excellent antibiotic producers in vitro (Baker and Snyder 1965). Therefore many of these soil microorganisms are ideal candidates for biological control agents (BCA) in sustainable agricultural production systems. The concept of how biological control

of soil-borne pathogens works was articulated at the 1963 international symposium entitled 'Ecology of soil-borne plant pathogens—prelude to biological control' (Baker and Snyder 1965). Baker proposed that antagonistic microorganisms could compete with pathogens, in particular through the production of antibiotic compounds. In soil, these antibiotics interfere with pathogen development, for example, during spore germination of fungal plant pathogens or prior to the onset of root infection.

Due to the complexity and extensive biological activity, the rhizosphere has proven to be an ideal location for inhabiting PGPR. Loosely defined PGPR are bacteria that can competitively colonize plant roots, and stimulate plant growth and/or reduce the incidence of plant disease. Mechanistically this can include: nitrogen fixation, phosphate solubilization, the production of phytohormones (such as auxin and cytokinin) and volatiles that function in plant growth stimulation (such as ethylene and 2,3-butanediol) (Ryu et al. 2003; Vessey 2003). Numerous studies have shown species specific effects of plants on the composition and relative abundance of microbial populations in the rhizosphere of crops and of cultivated native plant species (Smalla et al. 2001; Costa et al. 2006; Garbeva et al. 2008; Teixeira et al. 2010; Dias et al. 2012). For example, using DNA based culture-independent approach, a recent study of the phylogenetic composition of bacterial communities in the rhizosphere of three potato cultivars grown at two distant field sites, found that sequence abundance was the highest for Proteobacteria (46%), followed by Firmicutes (18%), Actinobacteria (11%), Bacteroidetes (7%) and Acidobacteria (3%) (Weinert et al. 2011). The bacterial families Streptomycetaceae, Micromonosporaceae, and Pseudomonadaceae showed the strongest response at the potato cultivar level. Therefore, the rhizosphere is a suitable resource to exploit for studies on bacterial diversity to develop plant host specific tools to enable sustainable agricultural crop production systems.

13.2.1 The Importance and Application of Streptomyces Diversity

Filamentous actinobacteria are considered important taxa within the rhizosphere microbiota (Benizri et al. 2001). Actinobacteria are able to influence plant development and protect the plant roots against soil borne plant pathogens. It is a diverse group consisting of 18 major lineages of bacteria, and its evolutionary divergence from other bacterial phyla is believed to be so ancient that it is difficult to identify their most closely related group (Ventura et al. 2007). The Actinobacteria are important to human medicine, agriculture and food production, due to their ability to interact with other organisms in the soil. The genus *Streptomyces* within the Actinobacteria produce numerous secondary metabolites that are used in human medicine as anti-infective, antitumor and immunosuppressant drugs (Challis and Hopwood 2003) and have complex developmental life cycles (Flårdh and Buttner 2009). *Streptomyces* use extracellular hydrolytic enzymes for the decomposition of organic matter in the soil that is recalcitrant to degradation by many other soil microbes (Loria et al. 1997). For these reasons *Streptomyces* spp. have gained a lot of

attention from applied and basic researchers for their importance in crop production systems and within the soil ecosystem (Aeron et al. 2011)

As stated earlier, *Streptomyces* spp. produce secondary metabolites such as geosmin (literally ‘earth smell’). Geosmin has no antibiotic activity, but gives the soil its characteristic smell, indicative of how widely distributed *Streptomyces* spp. are that inhabit the soil (Seipke et al. 2012). The adaptive significance of geosmin is unknown and presumably has an important role in the bacteria’s biology because of (i) production is a well-conserved trait in *Streptomyces* spp. (Hopwood 2007) and (ii) the gene responsible for geosmin production is well conserved among *Streptomyces* species (Gust et al. 2003). Regardless of the function of geosmin, the diversity of these secondary metabolites evolved through interactions among bacteria and other organisms, and their chemical diversity is yet to be discovered (Clardy et al. 2009). Members of the genus *Streptomyces* produce numerous secondary metabolites with antibiotic activity (Hopwood 2007). Due to potential interactions among *Streptomyces* spp. in the soil environment a stalemate has been created in an antimicrobial biological chemical arms race. Evolutionarily *Streptomyces* spp. carry genes that confer resistance to their own antibiotics to avoid suicide, these genes can spread to other soil bacteria and to pathogenic bacteria via horizontal gene transfer when the selection pressure (i.e., persistent exposure to antibiotics) is present in the soil environment (Cundliffe 1989; Aminov and Mackie 2007).

13.2.1.1 *Streptomyces* spp. in Bio-control of Phytopathogens and Plant Growth

Endophytic actinobacteria have been reported from a variety of plants. The most frequently isolated species belong to the genera *Microbispora*, *Nocardia*, *Micromonospora* and *Streptomyces* (Seipke et al. 2012). *Streptomyces* spp. are abundantly observed endophytes within the Actinobacteria (Sardi et al. 1992; Taechowisan et al. 2003). There is increasing evidence that streptomycetes are not just free-living soil bacteria, but can also form symbioses with plants. One of the reasons is due to its cosmopolitan distribution in soils ability to interact in beneficial, pathogenic or ammensal relationships with the roots of plants and other soil microbes. The beneficial endophytic *Streptomyces* spp. are growth promoting and plant protective in nature (Seipke et al. 2012).

There are several reports wherein *Streptomyces* spp. form protective mutualistic symbioses that feed the host, protect the bacteria, and in turn the bacteria produce antibiotics to protect the host, or the host resources from pathogens (Kaltenpoth et al. 2005). The filamentous morphology and ability to from spores enables *Streptomyces* spp. to colonize nearby roots and subsequently directly penetrate plant cells so as to gain entry into the host leading to endophytic and pathogenic phenotypes (Coombs and Franco 2003a, b; Franco et al. 2007; Joshi et al. 2007). Members of the genus *Streptomyces* that did not cause disease have been isolated from surface-sterilized root tissues of healthy wheat plants (*Triticum aestivum* L.). Although the *Streptomyces* spp. found were similar, based on 16S rDNA gene

sequences, to plant pathogenic *Streptomyces* spp. isolated from infected potato tubers. Additionally, several isolates exhibited high 16S rDNA gene sequence homology to *Streptomyces caviscabies* and *S. setonii* (Coombs and Franco 2003a, b). These isolates lacked the ability to produce the associated plant toxin essential to cause disease (Coombs and Franco 2003a, 2003b). This could suggest that when the soil is rich with resources and conducive to a transition to a mutualistic lifestyle the genes important in plant pathogenicity become non-essential and are thus lost to the environment via horizontal-gene-transfer (Coombs and Franco 2003a, 2003b).

Streptomyces spp. that cause plant disease are rare and comprise only a few well-studied species including: *Streptomyces turgidiscabies*; *S. acidiscabies*; *S. scabies* and *S. ipomoeae* (Loria et al. 1997). *Streptomyces* spp. as plant pathogens have a broad host range of plants, but are mostly known for their ability to cause necrotic scab-like lesions (Fig. 13.1) on economically important root and tuber crops such as potato. Scab-causing streptomycetes colonize root structures and subsequently penetrate plant cells directly and grow both inter- and intra-cellularly within the plant host (Joshi et al. 2007). The success of *Streptomyces* spp. as pathogens is linked to their ability to manipulate the plant host by producing a suite of plant toxins, secreting proteins and phytohormones to manipulate host physiology (Seipke et al. 2012).

Pathogenic *Streptomyces* spp. have one or more genes that are associated with pathogenicity (*nec1*, *txtAB*, and *tomA*). *TxtAB* is the gene (operon) responsible for the biosynthesis of the plant toxin thaxtomin, which is the main factor for common scab symptom expression in potato. *Nec1* and *tomA* are also associated with pathogenicity and virulence, although their function is a source of debate. Pathogenicity ‘islets’ can be located in distinct locations or clustered in the same region of the genome, depending on the species. Strain DS3024 (Hao et al. 2009), an Idaho strain (Wanner 2007), and two other strains (Wanner and Haynes 2009) have been identified that are genetically distinct from other known pathogenic *Streptomyces* spp. and lack *nec1*. Pathogenic *Streptomyces* spp. missing both pathogenicity-associated genes *nec1* and *tomA* have also been found (Wanner and Haynes 2009).

The commercially available biocontrol rhizobacterial strains of *S. griseoviridis* K61 (Mycostop; AgBio development) (Paulitz and Bélanger 2001; Schisler et al. 2004) and *Streptomyces lydicus* (SipCamAdvan LLC). For example *S. lydicus* in a commercial formulation, reduced the presence apothecia and sclerotia of *Sclerotinia sclerotiorum* (White Mold on soybean) in potted soil by 100 and 29.6%, respectively (Zeng et al. 2012b). Additionally experimental field trials with commercial formulations of *S. lydicus* reduced the disease severity index by 43.1 % and sclerotia by 90.6 % in soil (Zeng et al. 2012a).

13.2.2 The Importance and Application of *Bacillus* spp. Diversity

The genus *Bacillus* within the phylum Firmicutes contains over 88 species that have been cultured from the soil (Fritze 2004), and recent attempts to recover novel isolates have identified more species (Heyrman et al. 2004, 2005). Recent reports



Fig. 13.1 Lesion type on potato (*Solanum tuberosum*) tubers infected with common scab caused by *Streptomyces* spp., **a** superficial discrete; **b** coalescing superficial; **c** raised discrete; **d** raised coalescing; **e** pitted discrete; **f** pitted coalescing surface area of tuber covered with surface and pitted tuber lesions

indicate that genus *Bacillus* has 277 species with 7 subspecies (www.bacterio.net/index.html) The utility of *Bacillus* spp. as BCAs comes from their ability to generate a cadre of bioactive metabolites with a wide-spectrum of activity, among which are three families of cyclic lipopeptides (CLPs). These CLPs potential activity in biological control of plant pathogens is due to surface-active properties. The CLPs are classified into the surfactin, iturin and fengycin families, (Banat et al. 2000; Singh and Cameotra 2004). The CLPs have direct antagonistic activity against plant pathogens. The surfactins have haemolytic (Dufour et al. 2005), antiviral (Kracht

et al. 1999), antimycoplasma and antibacterial (Huang et al. 2007) activities but are not active against plant pathogenic fungi (Ongena et al. 2009). Similarly the iturins are also strongly haemolytic, but in contrast to surfactins display a strong in vitro antifungal action against a large variety of yeast and fungi, and limited antibacterial and no antiviral activities (Hiradate et al. 2002; Yu et al. 2002). The fengycins seem to have intermediate activity, less haemolytic than iturins and surfactins but they still retain a strong fungitoxic activity, specifically against filamentous fungi (Vanittanakom et al. 1986) and show some bacteriostatic effect against *E. coli* (Huang et al. 2007).

In addition to direct antibiotic activities, the surface-active properties of CLPs enables *B. subtilis* to effectively colonize plant roots, a prerequisite for plant growth promotion (Ongena et al. 2009). The surfactin family of CLPs can increase the surface area and bioavailability of hydrophobic water-insoluble substrates, heavy metal binding, bacterial pathogenesis, quorum sensing, motility and biofilm formation (Ron and Rosenberg 2001; Mulligan 2005). Many plant-associated bacteria have evolved and behave as structured communities called biofilms (on solid surfaces) or pellicles (at air/liquid interfaces) that adhere to root and on soil particle surfaces (Danhorn and Fuqua 2007). A community of bacteria working as a collective are motivated by common interest, as such microcolonies within the biofilm are sites that bacteria communicate with each other (quorum sensing) and act in a coordinated manner (Ongena et al. 2009). When resources in the soil are limited and mobility is required, surfactin and mycosubtilin can function in flagella-independent surface motility of *B. subtilis* (Kinsinger et al. 2003; Leclère et al. 2005). However, *B. subtilis* strain A1/3 only uses surfactin to form biofilms and pellicles indicating that surfactins may still serve specific developmental functions (Hofemeister et al. 2004).

13.2.2.1 *Bacillus* spp. for Biological Control

Members of the genus *Bacillus* are among the most widely utilized beneficial bacteria as BCAs for the management of diseases of economic crops. *Bacillus*-based products represent about half of the commercially available bacterial BCAs (Fravel 2005). Commercially available biocontrol rhizobacteria include *B. subtilis* strains GB03 (Kodiak; Gustafson), MBI 600 (Subtilex; Becker Underwood), QST 713 (Serenade; AgraQuest), *B. pumilus* strain GB34 (YieldShield; Gustafson), *B. licheniformis* strain SB3086 (EcoGuard; Novozymes), a mixture of *B. subtilis* strain GB122 and *B. amyloliquefaciens* strain GB99 (BioYield; Gustafson) and several *Bacillus* spp. (yield-increasing bacteria in China) (Paulitz and Bélanger 2001; Schisler et al. 2004). These BCAs are applied in commercial formulations as dry products (granules or powders), cell suspensions (with or without microencapsulation), or as seed treatments (Schisler et al. 2004). For more detail see Maheshwari (2013).

The mode of action of these BCAs in the control of soil-borne plant diseases is associated with CLP activity. The CLP iturin A produced by *B. subtilis* strain RB14 suppressed damping-off disease of tomato caused by *Rhizoctonia solani* (Asaka

and Shoda 1996). Additionally for control of post-harvest diseases, *B. subtilis* strain GA1, effective in producing three families of CLPs from a spectrum of fengycins, protected wounded apple fruits against gray mold disease caused by *Botrytis cinerea* (Toure et al. 2004). The fengycins were effective in providing disease control of fruits with CLPs-enriched extracts and by *in situ* detection of fengycins at inhibitory concentrations (Toure et al. 2004).

Although the CLPs are important antimicrobial secondary metabolites in the genus, *B. cereus* strain UW85 produces two fungistatic antibiotics, zwittermicin A and kanosamine that can contribute to the suppression of damping-off disease of alfalfa caused by *Phytophthora medicaginis* (Silo-Suh et al. 1994). Zwittermicin A may also control fruit rot of cucumber (Smith et al. 1993) and suppress other plant diseases (Silo-Suh et al. 1998). Therefore it is entirely possible within the biodiversity of bacteria in the soil, there are yet to be discovered *Bacillus* spp. capable of producing a suite of chemistries effective in sustainable disease management in commercial crop production systems.

Independent of the inoculums used (commercial or experimental), *Bacillus* populations in the rhizosphere tend to equilibrate and reach a threshold sufficient [10^5 – 10^7 colony forming units (CFUs)]/g of plant tissue fresh weight) to provide a beneficial effect locally to the host, likely not posing a threat to other non-pathogenic communities sharing the environment (Ongena et al. 2009). Additionally these *Bacillus* BCAs can co-produce different families of lipopeptides and are relatively benign to the environment and thus are classified as biopesticides by the United States Environmental Protection Agency (<http://www.epa.gov/pesticides/biopesticides/>).

13.2.3 The Importance and Application of *Pseudomonas* Diversity

Members of the genus *Pseudomonas* within the phylum Proteobacteria are rod-shaped Gram-negative bacteria. *Pseudomonas* spp. are characterized by metabolic versatility, aerobic respiration (some strains also have anaerobic respiration with nitrate as the terminal electron acceptor and/or arginine fermentation), one or several polar flagella for motility, and a high genomic G+C content (59–68%) (Haas and D  fago 2005). The term pseudomonads (*Pseudomonas*-like bacteria), is used to describe strains without taxonomic affiliation. Recently, a distinction was established between *Pseudomonas sensu stricto* (in the γ -subclass of Proteobacteria) and the genera *Burkholderia*, *Ralstonia*, *Acidovorax* and *Comamonas* (formerly called *Pseudomonas* but belong to the β -subclass). Certain pseudomonads are able to fluoresce due to the production of the pigment pyoverdine (pvd) (also known as pseudobactin). This is a large and heterogeneous group, *P. aeruginosa*, *P. putida*, *P. fluorescens* and *P. syringae* whose members include BCAs, animal, human and plant pathogens (Haas and D  fago 2005).

13.2.3.1 *Pseudomonas* Diversity in Biological Control of Plant Pathogens

Pseudomonas spp. function in bio-control of root diseases by producing six classes of antibiotic compounds such as phenazines, phloroglucinols, pyoluteorin, pyrrolnitrin, cyclic lipopeptides (all of which are diffusible) and hydrogen cyanide (HCN; which is volatile) (Haas and Keel 2003). Most bio-control strains of *Pseudomonas* spp. with a proven effect in plant bioassays produce one or several antibiotic compounds unrelated to typical siderophores (Haas and Keel 2003). The fluorescent pseudomonads produce Pvd or pseudobactin (Haas and Dèfago 2005), which functions as a diffusible, bacteriostatic or fungistatic antibiotic (Kloepper et al. 1980b, a; Scher and Baker 1982). In a deprived situation of iron in vitro, Pvd-producing *Pseudomonas* spp. inhibited the growth of bacteria and fungi with less potent siderophores (Kloepper et al. 1980b, a). Although Pvd-negative (Pvd⁻) mutants of fluorescent pseudomonads protect plants less effectively compared with the parental strains in some but not all, plant–pathogen systems tested under various environmental conditions, (Keel et al. 1989; Loper and Buyer 1991). A principal factor that influences bioavailability of iron in the rhizosphere is pH (Loper and Henkels 1997). Taken together, this suggests that Pvd-type siderophores may contribute in disease suppression, but alone they are not sufficient to account for the entirety of disease suppression that may explain why most fluorescent pseudomonads do not have bio-control activity (Haas and Keel 2003). Moreover siderophores are part of primary metabolism (iron is an essential element) and occasionally they behave as antibiotics (commonly considered to be secondary metabolites) (Haas and Keel 2003).

Commercially available bio-control rhizobacteria include *P. fluorescens*, *P. putida* and *P. chlororaphis* (Cedomon; BioAgri) (Paulitz and Bélanger 2001, Schisler et al. 2004). Root colonization is an important part of effective BCAs for plant protection by *Pseudomonas* spp. Thus *Pseudomonas* spp. can form microcolonies to communicate with each other and act in a coordinated manner. A root glycoprotein complex known as agglutinin can be involved in the short-term adherence of pseudomonads (Glandorf et al. 1994). Chemotaxis, flagellar mobility, lipopolysaccharide (LPS) structure, the outer membrane protein OprF and, to a lesser extent, pili are all important for competitive root colonization (Haas and Dèfago 2005). Once bio-control pseudomonads have moved and attached to the root zone, these microcolonies form in a few days in the grooves between epidermal cells. Subsequently other bacteria intermingle with pre-existing microcolonies. This development has been followed using differentially fluorescence-tagged *P. fluorescens* (Maurhofer et al. 1995). Typical cell densities range from 10^3 – 10^7 CFU cm⁻¹ of root, depending on the age and location of the microcolonies (Baker and Snyder 1965, Chin-A-Woeng et al. 1997). In the case of *Pseudomonas* spp. to protect plants from *Gaeumannomyces tritici* or *Pythium* spp. the critical density of colonization has been estimated at 10^5 – 10^6 CFU g⁻¹ of root. Thus, if plant roots are colonized by 10^8 – 10^9 culturable aerobic bacteria, it is estimated that bio-control pseudomonads likely represent 0.1–1 % of the culturable aerobic rhizobacterial populations under natural conditions (Bull et al. 1991; Raaijmakers et al. 1997, 1999; Haas and Keel 2003;

Chatterton et al. 2004). This 0.1–1 % of rhizosphere inhabiting microbe represents a wealth of unexplored bacterial diversity for potential BCAs.

13.3 Herbicide Resistant Cropping Systems

Many Soil-borne diseases are presumably kept in check most of the time by the natural soil microbial flora. Agricultural practices and other human activities are rapidly impacting the microbial flora prior to a basic understanding of a healthy soil microflora. Many of the current commercial production technologies may have long-term impacts on bacterial diversity vis-à-vis direct and indirect effects. One important tool in managed agricultural rotation systems is the availability of commercial herbicide-resistant crops. It is still unclear the environmental impacts on bacterial diversity due to the use of herbicide resistant crop varieties. Moreover the full range of resistance or sensitivity to the widely used herbicide glyphosate within the soil microbial community is also not known.

Since its commercialization, the herbicide glyphosate (N-phosphonomethyl glycine) has become the most popular herbicide world-wide, due in large part to the introduction and wide scale adoption of transgenic glyphosate-resistant (GR) crops (Duke et al. 2012). An unwanted side effect is the evolution of GR weeds species (Heap 1999), and naturally glyphosate-tolerant weed species (Johnson et al. 2009), which result in increased application rates and numbers of applications of glyphosate and other herbicides, per growing season in GR cropping systems (Duke et al. 2012). The target site, 5-enolpyruvylshikimic acid-3-phosphate synthase (EPSPS) of the glyphosate is found in green plants, and a limited number of microorganisms. EPSPS is an enzyme required for synthesis of the essential aromatic amino acids phenylalanine, tyrosine and tryptophan (Duke et al. 2012). Bacteria from bulk soil can influence the persistence of glyphosate and its metabolites. Furthermore bacteria in the rhizosphere can also influence uptake of soil minerals by plants. To evaluate the impact of glyphosate and its metabolites on the diversity of bacteria in the rhizosphere the direct and indirect effects via processes mediated by plants on root symbionts and rhizosphere microorganisms requires consideration (Duke et al. 2012). Indirectly glyphosate may also alter the quantity and quality of root exudates (Kremer et al. 2005). Significant amounts of carbon are exuded from growing roots, and rhizosphere populations may be exposed to glyphosate through leaching from the soil surface and root exudation.

Glyphosate can block the synthesis of the aromatic amino acids phenylalanine, tyrosine, and tryptophan in some bacteria through inhibition of EPSPS. EPSPS can also cause accumulation and excretion of shikimate-3-phosphate and hydroxybenzoic acids in sensitive microorganisms (Fischer et al. 1986; Moorman et al. 1992). Moreover EPSPS bacteria display a wide spectrum of sensitivity to glyphosate varies and have been classified into two groups: sensitive (Class I) and insensitive (Class II) (Pollegioni et al. 2011).

A myriad of techniques have been used to investigate the effects of glyphosate on microorganisms in soil. Studies on community level responses employing techniques to measure microbial biomass and respiration show either no effect or a temporary inhibition of respiration from the application of glyphosate (Wardle and Parkinson 1990; Lancaster et al. 2006; Zabaloy and Gómez 2008; Haney et al. 2009). Conversely increasing glyphosate application rates corresponds to the increase of soil respiration.

The influence of glyphosate applications on soil microorganisms can be evaluated by assessing spatial and temporal changes in bacterial community structure in long-term studies where the cumulative impacts can be determined. In a 19-year study, there was no difference in microbial biomass, microbial respiration and N mineralization in soils after annual glyphosate application compared to an untreated control soil (Hart and Brookes 1996). In another long-term study, microbial communities associated with Ponderosa pine (*Pinus ponderosa*) forest soils receiving glyphosate treatment for understory vegetation control were compared to control treatments (understory cover at 25–100%) (Busse et al. 2001). After 9 to 13 years glyphosate had no effect on soil respiration, N mineralization, or microbial biomass.

Recently a study using a DNA sequencing based culture-independent approach evaluated the effect on microbial community structure from GR corn rhizosphere after pre-emergence treatment with no herbicide, glyphosate, or GTZ (a mixture of the herbicides acetochlor and terbuthylazine) (Barriuso et al. 2011). The glyphosate treatment resembled that of the control (no herbicide). It reduced actinobacteria relative to the untreated control and proteobacteria were relatively unaffected. The GTZ treatment reduced microbial diversity similar to the glyphosate or no-herbicide treatments. Conversely another study found a variable response on actinobacteria populations in the soil after one or five applications of glyphosate to soil without a crop, while proteobacteria were increased by glyphosate applications (Lancaster et al. 2010). Moreover, consistent with the increase in Proteobacteria populations, the concentrations of microbial fatty acid methyl-esters (FAME) from Gram-negative bacteria and other proteobacteria, actinobacteria, and acidobacteria were increased (Barriuso et al. 2011). Using a DNA sequencing based culture-independent approach, there was little effect on the abundance of actinobacteria, and acidobacteria when glyphosate was applied post-emergence to GR-corn, and roots were sampled 7 days after glyphosate treatment and just prior to harvest, but the rhizosphere community was most affected by year and field and with no effect based on time of sampling and herbicide application. Acidobacteria increased over time in both fields, while actinobacteria tended to decrease (Barriuso et al. 2011). Similar studies using FAME biomarkers examined the effects on microbial communities in the soil of two post-emergent glyphosate applications to GR soybeans grown in soil with and without a history of previous glyphosate use (Lane et al. 2012). Using FAME analysis as an indicator of microbial biomass after 7 days of its application, biomass was reduced in both soils. Higher level analysis of FAME profiles showed significant legacy effects of the soil (history of glyphosate vs. no-history of glyphosate use) on community structure, but application or sampling times had no effect on community structure. When the analysis was repeated fol-

lowing a second in-season glyphosate application, the community structure of the bulk soil differed from that of the rhizosphere, but two previous applications of glyphosate (pre- and post-emergence) showed no effect on FAME profiles.

Beyond the indirect effects on rhizosphere bacterial diversity discussed above other potential side effects of the wide spread adoption of GR crops in rotation systems need to be considered. For example in the case of *Streptomyces* spp. the majority are saprophytes that subsist on organic matter in the soil. The potential increase of pathogenic strains of *Streptomyces* in the soil may be from the excess inputs of carbon from excess plant material leftover from GR crops. Moreover the shikimic acid pathway responsible for production of EPSs and tryptophan in GR crops, with the latter important in the production of plant toxin thaxtomin in pathogenic *Streptomyces* spp. (Loria et al. 2008) may be more accessible in the environment. There remains the possibility that this may be one of the reasons a persistence and greater severity of potato common scab caused by *Streptomyces* spp. in areas where the soil environment is conducive and GR crops have been used for decades.

13.4 Plant Disease Suppressive Soil

The rhizosphere of soils that are characterized by transferable plant pathogen suppressiveness can be a good source of PGPR, although disease conducive-soils also contain PGPR. Efficient root colonization, a desirable trait for BCAs can be selected by isolating bacteria that remain attached to the root surface, or have penetrated into the intercellular spaces between the root epidermis and the cortex, after extensive washing of the roots (Kloepper et al. 1980b; Stutz et al. 1986). For example *B. amylo-liquefaciens* strain BAC03, isolated from potato common scab suppressive soil, displayed antimicrobial activity against potato common scab (*Streptomyces* spp.), and the potential to enhance plant growth (Meng et al. 2012). Extracts from pure cultures inhibited growth and caused abnormal hyphal development of *S. scabiei* (Fig. 13.2).

Recently there has been renewed interest in the phenomena of endemic suppression of soil-borne plant diseases, including exploration of the effects of crop species, soil management and soil type on disease management and bacterial diversity. Intrinsic disease suppression in agricultural soils has been explored in multiple pathogen-plant systems (Atkinson 1892; Walker and Snyder 1934; Menzies 1959; Cook and Rovira 1976). Soil-borne disease suppression has been found in cultivated potato crop system soils (Liu et al. 1995; Bowers et al. 1996) and appears to be related to changes in microbial populations of antagonistic *Streptomyces* spp. characterizing the native rhizosphere bacterial diversity in naturally disease suppressive soils offers a new perspective for managing native soil bacteria to control soil-borne plant disease. Disease control is largely attributed to biological interactions (e.g., parasitism and competition) between antagonistic microflora and pathogens mediated via antibiotic production and/or enzymatic activity (Boudreau and Andrews 1987; Kinkel et al. 2011). Earlier, Gupta et al. (2006) found chitinase mediated control of disease in term of antagonism by potential PGPR *Pseudomonas*.

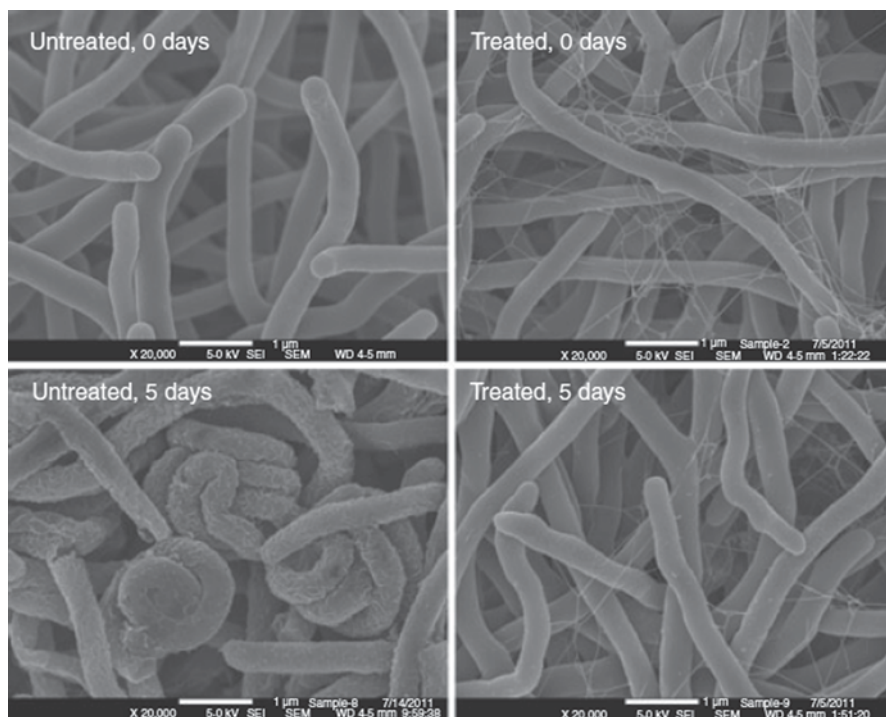


Fig. 13.2 Scanning electron microscopy (SEM) of mycelia morphology of *Streptomyces scabies* grown on yeast malt extract medium plates. Ten microlitres of sodium phosphate buffer (*untreated*) or ammonium sulphate precipitation (*treated*) was dropped onto a *Streptomyces scabies* 2-day old colony. Ammonium sulphate precipitation extracts from pure cultures inhibited growth and caused abnormal hyphal development inhibited *Streptomyces scabies*. Image is reproduced with permission, from Meng et al. © (2012) John Wiley and Sons © 2012 Michigan State University Journal of Applied Microbiology © 2012 The Society for Applied Microbiology. All rights reserved

Previous studies elaborated on disease suppression against *R. solani* AG2.2IIIB in sugar beet, *S. scabies* in radish and *Verticillium longisporum* in oilseed rape correlated positively with the presence of antagonistic *Lysobacter* spp. (Postma et al. 2008). Saraf et al. (2011) has reported a similar mechanism of disease suppression against *R. solani* with plant growth promoting rhizobacteria (PGPR) in sugarbeet. Moreover disease suppression against *S. scabies* (common scab) correlated with the high number of antagonistic bacteria, in particular with *Streptomyces* spp. and with the percentage of active fungi and number of antagonistic bacteria. (Schottel et al. 2001; Wiggins and Kinkel 2005; Postma et al. 2008). Take-all disease on wheat (*T. aestivum*) caused by *Gaeumannomyces graminis* var. *tritici* spontaneously decreases after several years of wheat monoculture and a severe outbreak of the disease (Berendsen et al. 2012). This phenomenon is known as ‘take-all decline’ that has been observed to be due to antagonistic fluorescent *Pseudomonas* spp. that produce the antifungal compound 2,4-diacetylphloroglucinol (DAPG) (Weller et al. 2002). The bacterial communities have been characterized in the rhizosphere of sugar beet

(*Beta vulgaris*) grown in *Rhizoctonia solani* suppressive soils using the PhyloChip high-density 16S ribosomal DNA oligonucleotide microarray (Hazen et al. 2010; Mendes et al. 2011). More than 33,000 bacterial and archaeal operational taxonomic units were present in the rhizosphere of plants grown in either suppressive or conducive soil. The rhizosphere bacterial community was dominated by members of the Proteobacteria (39%), Firmicutes (20%), and Actinobacteria (9%). The Gamma- and Beta- Proteobacteria, and Firmicutes were identified as the most dynamic taxa associated with disease suppression. Recent studies on a potato common scab-suppressive soil revealed a higher number of *Lysobacter* and of *Acidobacteria* (groups 4 and 6) in suppressive soil as compared to conducive soil (Rosenzweig et al. 2012). While, similar groups of bacteria dominated the rhizosphere in these diseases suppressive soil systems, it showed that it is possible that site-specific difference in bacteria community structure may have an impact indirectly on disease control.

Our knowledge of the biological factors contributing to the development of disease-suppressive soil is not well documented due to the complexity of microbe-microbe interactions (Menziez 1959; Hornby 1983; Alabouvette and Hornby 1990; Van Bruggen and Semenov 2000). With recent advances in DNA sequencing technologies and increase computational capacity there has been renewed interest to evaluate the factors implicated in endemic soil disease suppression, i.e., plant type, soil type and soil management that drive soil biology and specifically microbial community structure.

13.5 Current Methods to Study Bacterial Diversity

Researchers interested in bacterial diversity have frequently used molecular approaches employing cloning of 16S rDNA fragments obtained either after reverse transcription of rRNA (Ward et al. 1990; Weller et al. 1991), or after enzymatic amplification of DNA extracted from different habitats, including sediments (Grey and Herwig 1996), soil (Liesack and Stackebrandt 1992; Borneman et al. 1996), hot springs (Barns et al. 1994), and seawater (Giovannoni et al. 1990; Fuhrman et al. 1993). These studies have highlighted the wealth of microbial diversity, while simultaneously exposing limitations of traditional culture-based approaches to categorize this diversity. However, molecular cloning is time-consuming and labor intensive, and does not lend itself to multiple sample analysis. An alternative approach is the use of taxon-specific probes in dot-blot hybridization of extracted rRNA (Stahl et al. 1988; Raskin et al. 1995) or whole cell hybridization (Amann et al. 1995). These techniques are limited due to their focus on microorganisms for which probes have been developed and can therefore overlook rare taxa and novel biodiversity.

Molecular analysis via PCR amplification of variable ribosomal gene regions encoding 16S rRNA by use of primers homologous to conserved regions is effective for studying bacterial communities in the soil (Muyzer and Smalla 1998). This, coupled with separation by electrophoresis of the PCR products in a polyacrylamide

matrix over a denaturing gradient is a technique used for comparative studies of microbial diversity (Heuer et al. 1997; Heuer and Smalla 1997; Nakatsu et al. 2000). The denaturing gradient is a chemical gradient containing urea and formamide in denaturing gradient gel electrophoresis (DGGE) (Myers et al. 1985) or a temperature gradient in temperature gradient gel electrophoresis (TGGE) (Riesner et al. 1989). DNA fragments of the same length but of different sequences can be separated using either method. Both techniques have been shown to be equally effective, yielding, for example, comparable fingerprints of bacterial community diversity inhabiting the rhizosphere and phyllosphere of transgenic and non-transgenic potato plants (Heuer and Smalla 1997). DGGE has been used successfully to profile community complexity of a microbial mat and bacterial biofilms (Muyzer et al. 1993; Ferris et al. 1996, 1997; Santegoeds et al. 1996; Ward et al. 1996; Ferris and Ward 1997). Compared to other fingerprinting methods, DGGE and TGGE patterns allow subsequent in-depth analysis of bands of interest by sequencing or probing (Muyzer et al. 1993, 1995; Teske et al. 1996). However, typically only major constituents of an analyzed microbial community are represented as prominent bands in a DGGE fingerprint (Heuer and Smalla 1997) and rare taxa may not be reliably represented in a DGGE fingerprint. Thus, less abundant taxa, even if they represent functionally significant members of a microbial consortium, may be missed using this molecular approach.

Until recently, the ability to study the soil microbial community in a comprehensive manner was limited by the extreme size and the predominance of nonculturable microbes in soil communities (Pace 1996). Therefore, little or nothing is known about the billions of microorganisms that colonize the root-microbe-soil interface in disease suppressive fields. However, recent advances in sequencing technology to study microbial diversity (Margulies et al. 2005) provide the opportunity to explore the composition, structure, and diversity of bacteria important in sustainable agricultural crop production in greater detail than was previously possible.

13.5.1 High Throughput Next-Generation Sequencing

The recent accessibility of the next-generation sequencing (NGS) platforms has enabled analysis of microbial community structure of the soil affordable (Huse et al. 2008; Liu et al. 2008). High-throughput NGS platforms are capable of producing approximately 10^6 sequence reads of 400 bp in a single run, and provide greater depth than traditional DNA sequencing technologies and greater detection of rare species (Huse et al. 2008). Next-generation sequencing using the multiplex coded-primer (tag) approach enables high-throughput processing of multiple soil samples (Huse et al. 2007; Acosta-Martinez et al. 2008; Jones et al. 2009). This approach coupled with DNA amplification using conserved primers of phylogenetically informative regions of the 16S rDNA generates sufficient taxonomic information necessary for bacterial community diversity analysis (Yin et al. 2010). All high-throughput NGS platforms share common technological features, sequencing by synthesis and the technologies parallelize the sequencing process of clonally

amplified or single DNA molecules separated spatially within a flow-cell. In theory thousands or millions of sequences are processed in parallel simultaneously, and NGS platforms can generate hundreds of megabases to gigabases of nucleotide sequence output in a single instrument run, depending on the platform (Voelkerding et al. 2009). Moreover NGS platforms result in lower cost of DNA sequencing/base pair compared to traditional sequencing.

Using NGS, bioinformatics, computational biology and an ever-growing amount of sequence database information, it is possible to measure bacterial diversity, identify what bacteria are present and how abundant each organism is in a location and in or on a plant (Huse et al. 2008; Campbell et al. 2010; Manter et al. 2010; Sugiyama et al. 2010). The Ribosomal Database Project at Michigan State University (RDP; <http://rdp.cme.msu.edu>) provides rRNA related data, tools and analysis services to the research community (Cole et al. 2009). The data and services help the research community in the discovery and characterization of microbes important to soil biology and to the human microbiome. Additionally RDP's Functional Gene Pipeline/Repository (FunGene; <http://fungene.cme.msu.edu/>) contains a database of the most common functional genes and proteins important in soil ecosystems. Both RDP and FunGene offer tools for high-throughput gene-targeted sequence analysis (Iwai et al. 2009; Cole et al. 2011; Iwai et al. 2011).

13.5.2 Roche/454 Life Sciences Pyrosequencing

The 454 sequencing platform (<http://www.454.com>) combines pyrosequencing and emulsion PCR (emPCR). Initially a single-molecule DNA is amplified in microcompartments (droplet) of an oil and water solution with emPCR (Tawfik and Griffiths 1998). The single DNA template is ligated to oligonucleotide adapters and attached to a single primer-coated bead and forms a clonal colony of the PCR amplicon. Following emPCR amplification, the emulsion is disrupted, and beads containing clonally amplified template DNA are enriched prior to sequencing. The sequencing machine contains a sequencing plate that has many picoliter-volume wells. The enriched beads are separated by limited dilution, deposited into individual picotiter-plate wells, and combined with sequencing enzymes (Voelkerding et al. 2009). The pyrosequencing platform is based on chemiluminescent detection of pyrophosphate released during polymerase mediated deoxynucleoside triphosphate (dNTP) incorporation (Nyrén et al. 1993; Ronaghi et al. 1996; Ronaghi et al. 1998). Pyrosequencing uses luciferase to generate light for detection of nucleotides. The picotiter plate functions as a flow cell where the template DNA is immobilized and solutions of dATP, dCTP, dGTP, and dTTP nucleotides are sequentially added and removed from the reaction. Data output is in the form of a digital flowgram. In a single run the latest "Titanium" 454 chemistry in theory can generate approximately 1×10^6 sequence reads, with read lengths of ≥ 400 bases yielding up to 500 million base pairs (Mb) of sequence. One of the 454 sequencing platform's strengths is longer sequence read length, which facilitates de novo genome assembly (Pearson et al. 2007).

13.5.3 Life Technologies SOLiD Sequencing

The SOLiD (Supported Oligonucleotide Ligation and Detection) platform (<http://www.appliedbiosystems.com>) is a short-read technology based on sequencing by ligation similar to the 454 sequencing platform. The SOLiD platform is based on research developed for the re-sequencing of the *E. coli* genome (Shendure et al. 2005). Similar to the 454 platform DNA fragments are ligated to oligonucleotide adapters, attached to beads, and clonally amplified by emPCR. In lieu of a picoliter-plate, the flow-cell is a derivitized-glass surface. The clonal DNA beads, containing copies of the DNA molecule are deposited and immobilized on the glass slide for sequence analysis. A pool of all possible oligonucleotides of a fixed length (probes) are labeled according to sequenced position, and sequencing starts when oligonucleotides are annealed and ligated complementary to the adapter at the adapter–template junction (Voelkerding et al. 2009). The probes compete for annealing to the template sequences immediately adjacent to the primer. The preferential ligation by DNA for matching sequences results in a signal informative of the nucleotide at that position. After annealing, a ligation step is performed, followed by wash and removal of unbound probes.

13.5.4 Life Technologies Ion Torrent Sequencing

Life Technologies Ion Torrent sequencing platform (www.lifetechnologies.com) is unique among NGS technologies in that it based on complementary metal oxide semiconductor (CMOS) detection (Merriman et al. 2012). The sequencing chemistry is similar to 454 pyrosequencing, utilizing emPCR and sequencing-by-synthesis, with electrochemical detection of synthesis, and each such clonal reaction coupled to its own sensor, which are in turn organized into a massively parallel sensor array on a CMOS chip (Merriman et al. 2012). One nucleotide at a time is then added with solutions of dATP, dCTP, dGTP, and dTTP nucleotides. The DNA synthesis results in the release of a hydrogen ion (H^+) from the 3' OH incorporation site on the growing strand (Merriman et al. 2012). The release of a H^+ triggers a hypersensitive sensor in on the CMOS chip. The sensor detects changes in pH, and sequencing is performed by the sequential flow of nucleotide solutions, and monitoring for an incorporation signal via the sensor. The resulting signal is proportional to the number of released H^+ ions, hence the name Ion Torrent.

13.5.5 Illumina Sequencing

Unlike other NGS sequencing technologies the Illumina (<http://www.Illumina.com>) platform does not use emPCR. The Illumina sequencing platform is based on reversible dye-terminators. Similar to the SOLiD technology, a glass flow-cell

surface is used. The flow cell consists of an optically transparent slide with eight individual lanes on the surfaces of which are bound oligonucleotide anchors. DNA libraries are attached to a slide via primers with adapters complementary to the flow-cell anchors. Instead of emPCR a single DNA molecule is amplified to form local clonal colonies (bridge amplification). The bridge amplified arching “cluster” contains approximately 1000 clonal molecules (Voelkerding et al. 2009). Sequencing of the forward strands is initiated by hybridizing a primer complementary to the adapter sequences, one nucleotide (A, G, C or T) at a time is then added followed by addition of polymerase and a mixture of four differently colored fluorescent reversible dye terminators (Voelkerding et al. 2009). The dye terminators are subsequently chemically removed from the DNA, allowing the next cycle to proceed and non-incorporated nucleotides are washed away. A digital camera takes images of the fluorescently labeled nucleotides. Unlike pyrosequencing, the DNA can only be extended one nucleotide at a time. The Illumina sequencing technology as well as other NGS platforms have developed “paired-end” sequencing strategies to sequence both ends of template molecules which provides positional information to facilitate alignment and assembly, especially for short reads (Korbel et al. 2007; Campbell et al. 2008).

13.6 Conclusion

The exploitation of bacteria diversity in the rhizosphere for plant disease management will likely become a more pervasive tool in commercial crop production. Soil-borne diseases, such as late blight, responsible for the Irish potato famine of 1845–1849 (Fry and Goodwin 1997) caused by *Phytophthora infestans* (Mont.) de Bary (1876) have had transformative historical and social impacts. Technical advances in crop production make catastrophic events unlikely in this day and age in the developed world, yet soil-borne pathogens continue to pose a significant threat to modern crop production and hence food supplies, and their damage potential should not be overlooked (Hewitt 1998). With the withdrawal of many of the available chemistries for disease control, the development of sustainable biologically-based control strategies in plant production is more urgent than ever, and will further advance the capability of international agriculture to address critical needs in areas such as bio-energy, climate change, loss of agricultural land, and increasing global competition. The functional redundancy of bacteria diversity in the soil could provide activity via the added effect of a multitude of bacteria in the soil similar to a phalanx on the battleground. Ideally with the equipose of conventional and sustainable plant disease management the possibility of probiotic rhizosphere engineering could be a real possibility. There still remains the societal, economic and scientific barriers to overcome in order for the widespread adoption of BCAs in commercial crop production systems.

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